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LONGITUDINAL AND TRANSVERSE DIVISION IN TWO CLOSELY RELATED FLAGELLATES

L. R. CLEVELAND

(From the Biological Laboratories, Harvard University, Cambridge, Massachusetts)

The two flagellates considered in this paper are closely related morphologically, and are probably of common origin; yet one divides longitudinally in a typical flagellate manner, and the other transversely, as in ciliates. In the latter, the anterior daughter cell retains the parent extranuclear organelles, while the other daughter develops new organelles at the posterior end. This organism may represent the beginning stage in the development of a new group of flagellates.

The chromosomes of both organisms are interesting because they are large, two in number (except in rare instances), clearly differentiated in size, persist through the interphase, show structural details plainly, divide in the telophase, and are unmistakably moved to the poles by the extranuclear chromosomal fibres.

The achromatic figure is also interesting because the fibres composing it are as distinct as the flagella which the organisms possess, and it is plain that they have the same individuality as the flagella. They arise from the centrioles, which follow the flagellar bands, and are sometimes so intermingled with the flagella that they can be distinguished from them only by their function in nuclear division.

Eleven species of *Spirotrichonympha* have been described from termites but, with the exception of *S. polygyra*, the species described by Cupp (1930), the descriptions are so brief that it is impossible to determine how many species are valid. And the description of Cupp left unanswered many vital questions concerning the structure and behavior of extranuclear organelles and the processes of nuclear division. Further, an examination of the protozoa of *Kalotermes* (*Paraneotermes* Light) *simplicicornis* (Banks), the termite harboring the *S. polygyra* described by Cupp, shows that her description dealt with two distinct organisms, and is therefore not only inadequate but, in many respects, inaccurate. * However, it is not surprising that Cupp overlooked the fact that she was dealing with two organisms, because

they are so much alike in some respects—and yet so different in others. If the number of flagellar bands is not considered, their differences may be seen only when the processes of cell division are studied. These processes Cupp states she did not understand because of failure to find a sufficient number of successive stages.

An examination of the interphase flagellates of this termite shows that the number of organisms with two spiral flagellar bands is about equal to the number with four spiral flagellar bands. This fact Cupp noted, but she supposed that those with two bands were recently divided four-banded forms which would soon develop two new bands. But the number of two-banded forms is just as great in preparations without dividing organisms as in those with them. This alone indicates that the two-banded forms are not young or developing four-banded forms.

There are also forms with three, five, and six spiral flagellar bands, but these are very rare in comparison with the two- and four-banded forms. In all hypermastigote flagellates occasional irregularities occur in the flagellated areas, and therefore one should expect to find a few organisms, when countless thousands are studied, whose spiral flagellar bands likewise deviate from the normal numbers. These abnormalities are probably due, at least in part, as unpublished observations on *Barbulanympha* show, to exceptional behavior of the centriole, the organelle from which the flagellated areas arise; sometimes, one centriole fails to produce another from itself during cell division, in which case one daughter cell gets only one centriole while the other gets two; and at other times, one centriole produces two new centrioles instead of one from itself, thus giving one daughter three centrioles and the other two.

Organisms with one flagellar band also occur, and in preparations containing many dividing individuals they are present in greater numbers than the forms with three, five, and six flagellar bands; some of these are probably the result of irregularities in the behavior of the centriole, but most of them, as will be made clear presently, are posterior daughters produced in the transverse division of the body of the forms with two spiral flagellar bands.

Even though Cupp considered the forms with two spiral flagellar bands to be identical with those having four bands, her species, *S. polygyra*, is valid for the forms with four bands because she considered four to be the normal number of bands. It is only necessary to give a name to the form with two bands. This, however, is not an easy task, for its relationship to previously described species and genera must be considered from three angles: should it be placed in

any of the three genera which possess two flagellar bands; should *Spirotrichonympha*, which possesses four flagellar bands, be amended so as to receive it; or should a new genus be erected for it. Except for the fact that it has two instead of four flagellar bands, it is indistinguishable from *S. polygyra* when only interphase extranuclear organelles are considered, and its interphase nucleus does not differ greatly from that of *S. polygyra;* the four chromosomal coils are in two sheaths, while in *S. polygyra* each of the four chromosomal coils is in a separate sheath. This difference results from the failure of the sheaths containing the daughter chromosomal coils to separate in the late telophase of the two-banded form. But when the processes of cell division, particularly the behavior of the centrioles, the direction of the achromatic figure, and the plane of cytoplasmic division, are considered the two-banded form is so different from *S. polygyra* that one wonders whether it is desirable to place it in the same genus with *S. polygyra*. On the basis of the chromosomes alone, no one familiar with hypermastigotes, especially those with spiral flagellar bands, would hesitate to separate *S. polygyra* generically from *Macrospironympha*, *Leptospironympha*, and *Spirotrichosoma*, the previously described forms with two spiral flagellar bands, for *S. polygyra* has two rod-shaped chromosomes, while *Macrospironympha*, *Leptospironympha*, and *Spirotrichosoma* each has a fairly large number of V-shaped chromosomes. But the two-banded form under consideration also has two rod-shaped chromosomes which, like those of *S. polygyra*, are clearly differentiated morphologically into a short one and a long one; and it, too, should be separated from the previously described genera with two spiral flagellar bands. It is plainly a question, then, of whether one should separate *S. polygyra* and the two-banded form generically on a basis of four clear-cut differences; number of flagellar bands, behavior of centrioles, direction plane of the achromatic figure, and the plane of cytoplasmic division. Aside from the number of flagellar bands, the differences are largely physiological and the present tendency is not to place organisms in separate genera on this basis. This leaves for consideration only the number of flagellar bands. If the extranuclear organelles other than the spiral flagellar bands of *S. polygyra* and the two-banded form were at all different, the two organisms should be separated generically, but it has been impossible, after using various fixatives and stains, to detect a single difference. Hence, I believe the two organisms are more closely related than the number of flagellar bands indicates. In other words, the number of flagellar bands in this case does not represent a fundamental difference, and since it doesn't, it should not be used to separate the two organisms generically.

In view of the facts presented in the discussion above, the hypermastigote flagellate with two spiral flagellar bands in *Kalotermes* (*Paraneotermes* Light) *simplicicornis* (Banks) is placed in the genus *Spirotrichonympha* Grassi and Foa, 1911 and the species *bispira*, the species being new. In order to conserve space and to bring out more clearly the similarities and differences between *S. polygyra* and *S. bispira*, the morphology and processes of cell division in the two species are described in one paper.

I am indebted to Professor S. F. Light for a generous supply of the termites used in this study, to Miss Jane Collier for technical and research assistance, to Miss Dorothy G. Harris for making the drawings, and to the Penrose Fund of the American Philosophical Society for financial assistance. It was necessary to make and study eleven hundred permanent preparations before obtaining the information presented.

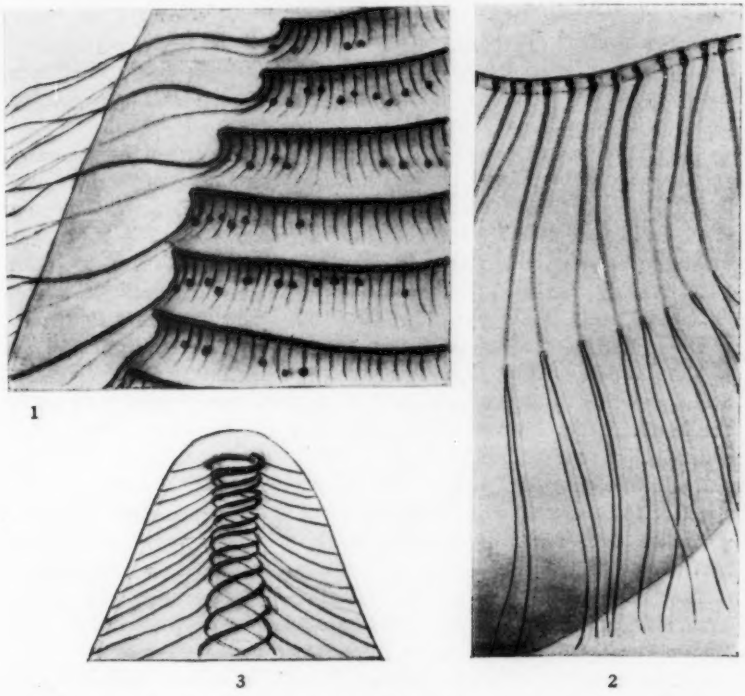
SPIROTRICHONYMPHA POLYGYRA

Morphology

In ten individuals the body ranged in length from 63 to 112 microns with an average of 81; the width at the widest portion ranged from 25 to 60 microns with an average of 45; the distance of the greatest width from the anterior end averaged 56 microns; the distance from the anterior end to the nucleus averaged 21 microns; the transverse diameter of the nucleus averaged 11 microns; the distance from the posterior termination of the spiral flagellar bands to the posterior end of the body averaged 26 microns; the length of that portion of the body covered by the flagellar bands (the flagellated area) averaged 55 microns; the number of turns or spirals made by the flagellar bands averaged 45; the length of flagella from the surface of the body averaged 20 microns; the length of flagella from the basal granules to the surface of the body averaged 7 microns; the total length of flagella averaged 27 microns; the distance from the flagellar bands (straight line) to the surface of the body averaged 5 microns, and the width of a flagellar band is about 1 micron.

In the anterior end the spirals made by the flagellar bands are small and close together and do not vary much for a distance of 5 to 7 microns. Then they gradually become larger and farther apart until the mid-region of the body is reached. From this point on to their termination there is little variation in their diameter and in the distance between them (Fig. 1). They turn sharply at their point of origin in the anterior end, and this gives them a ring-like appearance when viewed vertically. When viewed laterally at their point of

origin, they may be seen to originate in two groups, two in each group (Text-fig. *A*, 3). In other words, there are two points from which the bands grow out posteriorly, and two of the bands arising from each point lie side by side for a short distance. Sometimes, just before, but usually shortly after, cytoplasmic division the process of duplicating the flagellar bands begins. It cannot begin earlier, as in



TEXT-FIG. *A*. Details of portions of *Spirotrichonympha polygyra*. 1. Surface view of spiral flagellar bands. × 3000. 2. Flat surface of a band, showing rows of basal granules and the flagella that arise from the granules. × 4800. 3. Anterior end, showing how the flagellar bands begin to spiral. × 4800.

some related genera, because the four bands of the parent cell must unwind first and distribute two bands to each daughter, a process that is not completed in this flagellate until just before cytoplasmic division (Figs. 28–37). If the two new or daughter bands were to grow out before the unwinding of the parent bands was completed or nearly so, they would become entangled with the parent bands and

could never take their places besides them to form the interphase organism with four spiral flagellar bands.

When viewed laterally, depressions may be seen between the bands as shown in Text-fig. *A*, 1. In other words, the bands appear to be on ridges. This impression is probably produced by the ectoplasmic layer, which extends from the surface of the body to the bands, pushing inward between the bands. The flat surface of the flagellar bands does not lie parallel with the antero-posterior axis of the body as do the bands in *Macrospironympha*, but lies at an angle, the posterior margin of the band being nearer the surface of the body than the anterior, so that it is only when the bands are flattened that views of them as shown in Text-fig. *A*, 2 may be obtained.

The basal granules, from which the flagella arise, lie in rows antero-posteriorly directed across the flagellar bands, with two granules in each row (Text-fig. *A*, 2). The two flagella that arise from the granules in each row adhere to each other from their point of origin until they reach the surface of the body (Fig. 1, Text-fig. *A*, 1, 2). After leaving the band, the flagella extend posteriorly almost to the next band, from which point they turn somewhat abruptly and extend to the surface of the body, slanting posteriorly as shown in Fig. 1.

The large single axostyle is a hyaloid structure containing many fibres, extends from the anterior end through the central portion of the body, becomes wider as it approaches the nucleus which it surrounds, then smaller, enlarges again near the posterior end of the body, and after leaving the body tapers to a fine point. In the portion anterior to the nucleus the axostylar fibres are grouped close together, so that a few of them sometimes retain a small amount of stain in individuals suitably destained for nuclear details. This portion was mistaken for a centriole by Cupp (1930). By varying the destaining time, all degrees of destaining of the axostyle may be obtained, ranging from a heavily stained structure to one with no stain at all.

The so-called parabasals differ greatly in appearance from those of most genera of flagellates, but their reaction to fixatives and stains is the same, and I presume they should be regarded as parabasals. Instead of being long and slender, they are spherical and follow the spirals of the flagellar bands from a point near the posterior border of the nucleus to the termination of the bands (Fig. 1). They are probably attached to the bands since they follow them so regularly and since they retain their relationship to the bands when the bands and the body are broken up by drastic treatment; but it has been impossible to see a connection between them and the bands.

There are other structures, overlooked by Cupp and others who have studied *Spirotrichonympha*, which follow the flagellar bands just

as closely as the parabasals and which are clearly connected to the bands by fine threads, each structure being connected by a single thread. These structures are considerably smaller than the parabasals, stain deeply following fixatives which do not render the parabasals stainable, and follow the bands a considerable distance anteriorly beyond the termination of the parabasals (Fig. 1). In every individual, they terminate at practically the same point, namely halfway between the mid-portion of the nucleus and the anterior end; and they extend posteriorly to the termination of the bands. But, unlike the parabasals, they lie closer together along the bands in the region of the nucleus and anterior to the nucleus than elsewhere. They, like the parabasals, probably arise from the bands, since various stages in their growth may be noted in new bands which are increasing in length posteriorly.

Mitosis

The chromosomes are duplicated in the late telophase (Fig. 15), and as a result of this each interphase nucleus contains four chromosomes (Figs. 3–7). Each chromosome contains a distinct coil within a sheath and is anchored to the anterior margin of the nuclear membrane by an intranuclear chromosomal fibre. These ends of the chromosomes are referred to as the anterior ends or as the attachment ends, while the opposite ends, which are attached either to a chromatin nucleolus or to portions of it, are referred to as the posterior ends. The portions of the chromatin nucleolus attached to the chromosomes vary considerably in size and shape (Figs. 3, 5); sometimes a single piece may be attached to all the chromosomes (Fig. 4); at other times a piece may be attached to each pair of chromosomes (Fig. 8). The daughter chromosomes are usually twisted around each other, the degree of twisting varying considerably in different nuclei. When all the chromosomes are attached to one nucleolus, they are twisted little if any, and in such nuclei the direction of the chromosomal coils may be followed plainly from one end of the chromosomes to the other (Fig. 4). It is also possible in many of the other nuclei where the chromosomes are each attached to a portion of the chromatin nucleolus and hence are permitted to twist considerably, to follow the coils clearly from the anterior to the posterior end of the chromosomes and to see that the coil sometimes reverses its direction in an individual chromosome (Fig. 3); and when each pair of daughter chromosomes is attached to a portion of the chromatin nucleolus, as illustrated in Fig. 8, the direction of the coils may be determined at a glance because they are so plain. A coil may reverse its direction at any point in a chromosome, and the coils of daughter chromosomes may be directed

either alike or differently. Models of chromosomal coils, made of wires, show that the shifting of direction of coiling in a chromosome does not interfere with the coil splitting lengthwise, as it does in the telophase, and separating to become two individual coils of daughter chromosomes.

The first step in cell division is the separation of the spiral flagellar bands at their anterior ends, two of the bands moving in one direction and two in the other; and as they separate they unwind, a process that is not completed until shortly before cytoplasmic division (Figs. 28–37). Almost immediately after the bands begin to separate and unwind, the achromatic figure begins to form between the two groups of separating bands (Figs. 28, 29). In most dividing organisms the fibres of the achromatic figure, like the flagella, appear to arise directly from the flagellar bands about midway between the nuclei and the anterior ends of the cells, but in a few individuals—especially those distorted by drastic treatment—a fine, darkly staining line may be seen following one band of each group (of two bands). This line is the elongate centriole from which the fibres of the achromatic figure arise (Fig. 31). It should be noted here that in other genera of the Spirotrichonymphidæ previously studied (Cleveland, Hall, Sanders, and Collier, 1934) two elongate centrioles follow the spiral flagellar bands from their point of origin at the anterior ends of the cells for a considerable distance posteriorly, but their posterior or distal ends, which produce the achromatic figure, are free of the bands. In *Pseudotrichonympha*, a hypermastigote without flagellar bands (Cleveland, 1935), the unusually long centrioles adhere to the inner margin of the rostral portion of the flagellated area, although this, comparatively speaking, is a short portion of the centrioles; in *Barbulanympha*, a hypermastigote with two, short flagellated areas at the anterior end instead of flagellar bands, the centrioles which are about 40 microns in length, adhere to the lamella underneath the flagellated areas for a distance of 6 to 10 microns; and in *Trichonympha*, another hypermastigote without flagellar bands, the posteriorly directed elongate centrioles are free except for their point of origin where they are fastened to the lamella underneath the flagellated area. Thus, there are all degrees of anchorage of the centrioles to another organelle, ranging from *Trichonympha*, *Joenia*, *Joenopsis*, and other genera, where the anchorage is slight, through *Barbulanympha*, *Urinympha*, *Rhynchonympha*, *Eucomonympha*, *Teranympha*, *Staurojoenina*, *Macrospironympha*, *Spirotrichosoma*, and *Leptospironympha* to *Spirotrichonympha*, where it is complete. Without this series, the situation in *Spirotrichonympha* would seem unusual and would be difficult to understand.

The manner in which the achromatic figure arises from the centrioles that follow two of the four flagellar bands is similar to that in other hypermastigotes: Astral rays arise from the distal end of each centriole, meet, join, overlap, and grow along one another to form the central spindle (Figs. 28–31). As more rays overlap, the central spindle becomes broader; and as the rays increase in length, it becomes longer. However, a point is reached shortly after nuclear division when the central spindle ceases to broaden, and it soon begins to narrow, because the daughter flagellar-band centriole complexes, to which its ends are anchored, move in opposite directions and thus pull apart the astral rays that formed the central spindle, the last-joined astral rays being the first ones to pull apart (Figs. 33, 35). Finally, the central spindle is pulled apart and disappears (Fig. 36).

The early central spindle lies a considerable distance anterior to the nucleus (Figs. 28–30), but, as it increases in size and the anterior ends of the developing daughter cells move in opposite directions, it is gradually moved toward the nucleus, finally stretching directly across it (Figs. 31, 32). The direction of the central spindle in *S. polygyra* is crosswise to the long axis of the body, while in *S. bispira*, as will be described presently, it extends parallel with the long axis. Shortly before the central spindle comes in contact with the ever intact nuclear membrane as a result of its moving posteriorly, some of the astral rays become extranuclear chromosomal fibres by making contact with the intranuclear chromosomal fibres anchored in the nuclear membrane, and as the central spindle increases in length, due to the centrioles from which it arises moving in opposite directions, these extranuclear chromosomal fibres begin to move the chromosomes toward the poles, one short and one long daughter chromosome moving toward each pole (Figs. 9, 10). In order to conserve space and since the same situation exists in *S. bispira*, where it is illustrated, the extranuclear chromosomal fibres have not been drawn; nor has the relation of the central spindle to the nucleus and chromosomes been shown in most instances. However, the position of the central spindle and of the extranuclear chromosomal fibres can be determined in the drawings of the nuclei by the direction of the intranuclear chromosomal fibres; the extranuclear chromosomal fibres extend from the intranuclear chromosomal fibres in the nuclear membrane in the same direction as the intranuclear chromosomal fibres (Figs. 9–12); and the direction of the extranuclear chromosomal fibres indicates the position of the central spindle, since they and the fibres of the central spindle arise from the distal ends of the centrioles. The nuclei (Figs. 3–15) are all mounted so that their anterior surfaces point toward the top of the page.

Just before the chromosomes begin their poleward movement, they become considerably shorter and the turns made by their spirals become broader. These stubby chromosomes persist until shortly after nuclear division (Figs. 9–12); then they gradually elongate and their coils and sheaths split lengthwise, thus producing one short and one long pair of chromosomes which persist through the interphase (Figs. 13–15).

SPIROTRICHONYMPHA BISPIRA SP. NOV.

Morphology

In ten individuals the body ranged in length from 59 to 102 microns with an average of 81; the width at the widest portion ranged from 32 to 48 microns with an average of 40; the distance of the greatest width from the anterior end averaged 57 microns; the distance from the anterior end to the nucleus averaged 21 microns; the transverse diameter of the nucleus averaged 10 microns; the distance from the posterior termination of the spiral flagellar bands to the posterior end of the body averaged 27 microns; the length of that portion of the body covered by the flagellar bands (the flagellated area) averaged 54 microns; the number of turns or spirals made by the flagellar bands averaged 34; the length of the flagella from the surface of the body averaged 20 microns; the length of the flagella from the basal granules to the surface of the body averaged 7 microns; the total length of the flagella averaged 27 microns; the distance from the flagellar bands (straight line) to the surface of the body averaged 5 microns, and the width of a flagellar band is about 1 micron.

It is clear from these measurements that *S. bispira* differs from *S. polygyra* neither in the size of the body nor in the size of the organelles. And the interphase organelles of the two organisms do not differ in appearance and number except for the number of bands and the enclosure of each pair of daughter chromosomal coils in a common sheath in *S. bispira*, differences already noted. There is, however, a notable difference in the number of turns made by the flagellar bands, those of *S. polygyra* making 45 and those of *S. bispira* making 34. Here is a fact which in itself indicates that the two-banded and four-banded forms are distinct organisms; for, if the forms with two bands making 34 turns developed two more bands and these new bands followed the old ones, as they would have to do, the number of turns would be 68. The bands of *S. bispira* are slightly farther apart, especially in the anterior end, than those of *S. polygyra* (Figs. 1, 2). The description given for the axostyle, flagella, parabasals, and other extranuclear organelles of *S. polygyra* takes care of these organelles

in *S. bispira*, and we may proceed immediately with the description of mitosis.

Mitosis

The various processes connected in one way or another with mitosis and cell division in *S. bispira* are unusually interesting because they are so plain, because of their unique features, and because they differ decidedly from those of *S. polygyra*. There is one short and one long chromosomal sheath in the interphase nucleus and each sheath contains two chromosomal coils (Fig. 16). The division of the coils occurs in the late telophase (Fig. 27). Each coil is attached to the nuclear membrane by a fibre which is termed the intranuclear chromosomal fibre. Until the early anaphase, each coil is thus anchored to the anterior margin of the nuclear membrane (Figs. 16–20). (In all the drawings of nuclei the anterior margins are directed toward the top of the page.) The opposite or posterior ends of the chromosomes are all attached either to a single chromatin nucleolus or to portions of a chromatin nucleolus, as described in *S. polygyra*.

The first step in nuclear division is the division of the chromosomal sheath and the moving apart of the daughter chromosomal coils (Fig. 17). As the daughter chromosomes move apart, they frequently coil around each other (Figs. 17–19). This movement of the chromosomes is not poleward movement, but merely movement within the nucleus, and the daughters are probably interconnected during this period—although interconnections cannot always be seen—for later when they contract and straighten, just before poleward movement begins, interconnections may be seen plainly (Fig. 20); and these strands connecting the chromosomes are not broken until considerable progress in poleward movement has been made (Figs. 21–23). It should be noted here that poleward movement of the chromosomes is not to the right and left, as in *S. polygyra*, when the anterior end of the organism and the anterior surface of the nucleus are foremost, but is anterior and posterior (up and down). In other words, the chromosomes of *S. polygyra* move at right angles to the long axis of the cell, while those of *S. bispira* move parallel with the long axis. The reason for this will be clear when the development and function of the achromatic figure is explained.

So far as I know, there is no cell hitherto described where the behavior of the centrioles during the formation of the achromatic figure is the same as in *S. bispira*. There are two spiral flagellar bands in each interphase organism, but it has been impossible to determine whether a centriole follows one of these bands prior to the beginning

of cell division, because during division a centriole usually follows one band so closely that the centriole and the band appear as one structure. In other words, the centriole cannot be distinguished from the band, and its presence during division, with the exception of a few instances, can be demonstrated only by its function. The elongate centriole which follows the band during division may persist as an elongate centriole during the interphase, just as the centrioles of *Barbulanympha* and many other genera persist; it may degenerate except for its anterior portion in the late telophase, just as the centrioles of *Pseudotrichonympha* do; or there may be one short and one long centriole in the interphase as in *Trichonympha*. At present, I see no way to determine which possibility is correct. But, irrespective of whether the posterior portion of one centriole which follows a band during division degenerates or persists during the interphase, the manner in which it functions during cell division may be demonstrated clearly.

In order to facilitate the explanation of the processes concerned with cell division, it seems desirable to name and label some of the structures involved in the processes. The two interphase flagellar bands which persist and do not change position during cell division are designated *parent flagellar bands* (*p.f.b.*). In most organisms a centriole follows one of these bands from its point of origin at the anterior end of the cell for a distance of 4 to 5 turns or spirals, and these two structures are designated *centriole parent flagellar band* (*c.p.f.b.*). Occasionally the centriole does not follow the band for more than two turns before it separates from it and continues independently; then it is labeled *centriole* (*c.*). The earliest stage in cell division is the growth of a new flagellar band from the point of origin of the parent flagellar bands posteriorly. This new flagellar band has a centriole which, in the early stages of development, follows it for its entire length, and where the two follow each other they are designated *centriole new flagellar band* (*c.n.f.b.*). This new band soon breaks loose from its point of origin, becomes free, and migrates posteriorly; then the end which at one time was connected to the parent flagellar bands at their point of origin in the anterior end of the cell is designated the *anterior end* (*a.e.*); the other end which, as development progresses, elongates beyond the point where the centriole terminates is designated the *elongating end* (*e.e.*). As this new band elongates posteriorly from its point of origin, it develops basal granules from which *flagella* (*f.*) grow out. *Astral rays* (*a.r.*) arise from the distal portion of the centriole following the parent flagellar band and soon meet those that arise from the same portion of the centriole that follows the new flagellar band; when they meet, they join, overlap,

grow along one another and thus form the *central spindle* (*c.s.*). The centriole that follows one of the parent flagellar bands may be referred to as a *stationary centriole*, and the one that follows the new or free flagellar band as a *free centriole*, although it is not free in a sense because it is attached to the band. Similarly, the astral rays arising from the two centrioles may be referred to (Figs. 52, 53) as *astral rays* from *free centriole* (*a.r.f.c.*) and *astral rays* from *stationary centriole* (*a.r.s.c.*).

The formation of a new flagellar band—first attached at its point of origin, then free and migrating posteriorly while an achromatic figure is formed between it and one of the (stationary) parent flagellar bands—presents an interesting picture in the mechanics of cell division and renewal of extranuclear organelles. This migrating flagellar band, extending in length as it migrates, evidently has considerable difficulty in finding its way to the posterior end of the cell, for it is sometimes entirely outside the parent flagellar bands and sometimes partly outside and partly inside. Its movement is further handicapped because it is joined to one of the parent flagellar bands by the central spindle arising from its centriole and that of the parent flagellar band. But in most instances it eventually makes its way to the posterior end of the cell and, while so doing, picks up and carries with it two chromosomes, half of the nuclear membrane, and other nuclear materials. However, it sometimes fails to make this journey, and these instances are particularly interesting from the standpoint of the manner in which the achromatic figure is formed and its function in nuclear division. Two such instances are illustrated: in one (Fig. 52) the new band appears to be caught by the parent flagellar bands near its anterior end; in the other (Fig. 53) the new band appears to be lodged to the side and anterior to the parent flagellar bands. In the first instance, it is difficult to distinguish clearly astral rays arising from the centriole following the new band from the flagella that are arising from the new band, but the astral rays that are arising from the distal end of the centriole that follows one of the parent flagellar bands are long and plain and extend between the spirals of the parent bands to the nucleus (Fig. 52). In the second instance, astral rays may be seen clearly arising from the centriole following the new band; they extend inwardly and the flagella, for the most part, outwardly (Fig. 53). The astral rays that arise from the stationary centriole (*a.r.s.c.*) extend within the spirals to the nucleus. In neither instance is a central spindle formed, because the centrioles, from which the astral rays forming the central spindle arise, are so placed that the astral rays arising from one centriole cannot meet those arising from the

other. In both instances the chromosomes have the appearance of telophase chromosomes, yet the pairs of daughter chromosomes have not moved apart because the proper formation and function of the achromatic figure was upset by the position of the free centriole which adheres to the improperly placed new flagellar band.

The fact that the free centriole in this organism assumes almost every conceivable position during its posterior migration is responsible for the great variation in the appearance of the achromatic figure. For example, the central spindle of the achromatic figure may extend from the free centriole to the stationary centriole entirely within the parent flagellar bands (Figs. 49–51, 55); it may be entirely outside the parent bands (Fig. 47); one portion of it may be inside and the other outside (Figs. 43, 48); or, as is more often the case, it may be partly outside and partly inside (Figs. 42, 44, 45). The end of the central spindle adjacent to the stationary centriole is fairly constant in shape when the centriole follows the parent flagellar band closely, because it assumes the shape of that portion of the centriole-band complex from which the fibres arise, and hence is cylindrical (Figs. 50, 51); but when this centriole does not follow the band closely, as occasionally happens, there is nothing to direct the fibres into a cylindrical position and they merely extend posteriorly from the distal portion of the centriole (Figs. 41, 42, 45). The shape of the other end of the central spindle depends entirely on the position of the new flagellar band and the centriole following it. If the centriole and band lie in almost a straight line, the adjacent end of the central spindle is flat or nearly so (Figs. 47, 51); if they lie somewhat in a cylindrical position, the adjacent end of the central spindle is somewhat cylindrical (Figs. 44, 50); if they are zigzag or wavy, so is the adjacent end of the central spindle (Figs. 43, 45, 46, 49). The position of the free band and centriole may also be such that only a few of the astral rays arising from the stationary centriole join those arising from the free centriole to form a central spindle outside the parent flagellar bands, while the other astral rays arising from the stationary centriole extend between the parent bands to the nucleus (Fig. 46). Many variations in the numbers of fibres that join or fail to join occur.

The rôle of those astral rays which, by becoming extranuclear chromosomal fibres, function in the movement of the chromosomes to the poles is plain. These rays are longer than those which in other hypermastigotes perform the same function. This is because the nucleus lies farther away from the centrioles, the point of origin of the rays, than in most cells. In other words, the astral rays become long before they reach the nucleus. In some instances these extranuclear

chromosomal fibres may be followed all the way from their point of contact with the intranuclear chromosomal fibres in the nuclear membrane to the poles (Figs. 45, 55); in others they are lost among the fibres of the central spindle soon after they leave the nuclear membrane (Fig. 51). There can be no question regarding the fact that they carry one short and one long chromosome anteriorly and one short and one long one posteriorly, because, when the centrioles from which they arise are so situated in the cell that these fibres cannot reach the chromosomes, the chromosomes do not move apart and the nucleus does not divide.

The chromosomes are anchored to the anterior surface of the nuclear membrane until the anaphase, and if there were no interchromosomal connections between the daughters of the two pairs, two of the chromosomes would not move at all until the nuclear membrane began to elongate prior to pulling in two. The situation would be very simple, as is occasionally the case when the connections between the daughter chromosomes are broken in the prophase or metaphase: two chromosomes (a long one and a short one) move to the posterior surface of the nuclear membrane, the nuclear membrane elongates, pulls in two, and daughter nuclei are developed. But the more usual thing is for the connections between the daughter chromosomes to persist until the two chromosomes that are going to form a part of the posterior daughter nucleus are pulled to the posterior surface of the nuclear membrane (Figs. 21, 22). The connections between the posterior ends of the chromosomes are the last ones to break because the pull on the chromosomes is from their anterior ends. Incidentally, we have here an explanation of what produces the so-called equatorial plate stage in chromosomes; the pull on the daughter chromosomes from opposite directions (antero-posteriorly in this organism) causes them to take up a more or less central position within the nucleus. Variations of this stage, of which there are many in different organisms, result from the irregularity in the behavior of the connections between daughter chromosomes, those in some organisms breaking sooner than in others.

Cell Division

The type of cell division in *S. bispira* is so unusual that it deserves special attention. Shortly after the division of the nucleus and the disappearance of the achromatic figure, the new flagellar band, which now lies in the posterior end of the cell (Fig. 56), begins to arrange itself in the form of a spiral, the first portion to so arrange itself being the anterior end (Fig. 57). This is the end which in the early stages

of mitosis lies adjacent to the anterior ends of the two parent flagellar bands (Figs. 38, 39), the end which does not elongate and which is labeled *a.e.* in the illustrations of the formation and development of the achromatic figure (Figs. 39–55). It now becomes the anterior end of the daughter cell which is developing at the posterior end of the parent cell (Figs. 56–59). This new band soon becomes arranged in spirals from the anterior to the posterior end (Fig. 58). Meanwhile, both the posterior and anterior developing daughter cells begin to form new axostyles (Figs. 56–58). Transverse cytoplasmic division soon occurs, producing two independent daughter cells; one, the anterior daughter, obtaining a nucleus and all the parent extranuclear organelles intact except the axostyle; the other, the posterior daughter, obtaining a nucleus, the new flagellar band, the centriole adhering to the new band, a small amount of the parent cytoplasm, and the extranuclear organelles such as flagella, parabasals, axostyles, etc. developed before cytoplasmic division. In other words, all the extranuclear organelles of the parent cell except the axostyle, which is resorbed, are carried over into the anterior daughter, and the posterior daughter gets the new organelles. The axostyle surrounds the nucleus in such a manner that the nucleus probably could not divide if this organelle remained intact. So far as I know, the extranuclear organelles of flagellates are either resorbed and each daughter develops a new set of organelles or the parent organelles are distributed among each daughter. *S. bispira* shows a radical departure from either procedure, and one wonders if this is not the beginning of a new stage of evolution in flagellates.

After cytoplasmic division, the posterior daughter, which is considerably smaller than the anterior one, continues the development of its extranuclear organelles. The axostyle increases in length and breadth and the flagellar band extends posteriorly, forming spirals as it does so, although the spirals for some time are not perfectly arranged (Fig. 59). They soon become arranged more or less perfectly (Fig. 60), and presently another flagellar band begins to develop from the point of origin of the existing or first new band. This band is referred to as the second new band. It extends posteriorly and soon takes its place along the first new band, so that the posterior daughter cell now has two spiral flagellar bands (Fig. 61). These bands increase in length and finally become as long as the parent bands were prior to the beginning of the processes of cell division. As the bands increase in length, they form more flagella and parabasals, the axostyle becomes grown, and the posterior daughter is now indistinguishable from the parent cell from which it originated (Fig. 2). Meanwhile, the axostyle

of the anterior daughter completes its growth, and it, too, becomes indistinguishable from the parent cell from which it originated.

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EXPLANATION OF PLATES

The drawings were made with the aid of a camera lucida from material fixed either in Schaudinn's or Flemming's fluids and stained with hæmatoxylin.

PLATE 1

FIG. 1. *Spirotrichonympha polygyra.* Entire organism. Note four spiral flagellar bands. The flagella leave the bands in groups of two, and only two groups are shown from each band. The two adhering flagella extend posteriorly from their point of origin almost to the next band; then they turn and continue somewhat posteriorly to the surface of the body where they separate. The spherical parabasals follow the bands from a point just posterior to the nucleus to their termination. Other bodies, smaller than the parabasals, follow the bands from midway between the anterior end and nucleus to their termination. The axostyle extends from the anterior end around the nucleus to the posterior end of the body, terminating in a fine point slightly beyond the body. × 1600.

PLATE 2

FIG. 2. *Spirotrichonympha bispira* sp. nov. Entire organism. The extranuclear organelles differ from those of *S. polygyra* only in that there are two instead of four spiral flagellar bands. × 1600.

PLATE 3

Spirotrichonympha polygyra

The nuclei are mounted so that their anterior surfaces are directed toward the top of the plate.

FIG. 3. Interphase nucleus. There are four chromosomes. Each chromosome has a single coil lying within a sheath and is anchored to the anterior surface of the nuclear membrane by an intranuclear chromosomal fibre. The posterior end of each chromosome is attached to a portion of the chromatin nucleolus. There are two long and two short daughter chromosomes. The daughters of each group are somewhat coiled around each other. × 3800.

FIG. 4. Interphase nucleus. Both pairs of daughter chromosomes are attached to the chromatin nucleolus at their posterior ends; anteriorly they are attached to the nuclear membrane as explained in Fig. 3. × 3800.

FIG. 5. Interphase nucleus. Here the pairs of daughter chromosomes are considerably coiled around each other, the long pair lies over the short pair, and the posterior end of each chromosome is attached to a portion of the chromatin nucleolus. × 2400.

FIG. 6. Interphase nucleus more heavily stained than the three previous nuclei, so that other material in the nucleus besides the chromosomes stains and thus obscures slightly the arrangement of the chromosomes and their connections with portions of the chromatin nucleolus. × 3800.

FIG. 7. Interphase nucleus so heavily stained that chromosomal details cannot be seen. × 2400.

FIG. 8. Prophase nucleus. The daughter chromosomes are no longer coiled around each other. The coils within each chromosomal sheath are plain and their direction may be followed clearly. × 3800.

FIG. 9. Rather heavily stained nucleus. Each pair of chromosomes is short and stubby, the turns made by the coils within the sheaths are broader and closer together, and the daughter chromosomes are beginning to move apart. The chromosomal coils here and until the late telophase are not so distinct as in the interphase and prophase. The attachments between chromosomes and portions of the chromatin nucleolus will be lost presently, not to be renewed until the very late telophase. × 3800.

FIG. 10. The greatly shortened chromosomes have moved apart and a large one and a small one are preparing to line up on opposite sides of the nucleus. × 3800.

FIG. 11. Nucleus has elongated. One large and one small chromosome have been moved to the left side of the nucleus and the other two chromosomes are in the process of being lined up in the same manner. × 3800.

FIG. 12. The nuclear membrane has pulled in two and each daughter nucleus has a pair of dimorphic chromosomes. × 2400.

FIG. 13. Daughter telophase nucleus. Chromosomes are beginning to elongate and the coils within their sheaths are again distinct. × 2400.

FIG. 14. Daughter telophase nucleus. The chromosomal coils are in the process of duplicating themselves by longitudinal division. × 2400.

FIG. 15. Daughter telophase nucleus. The chromosomal coils have divided and the chromosomal sheaths have almost completed the process of longitudinal division. Connections are being made between chromosomes and portions of the chromatin nucleolus. × 2400.

PLATE 4

Spirotrichonympha bispira

The nuclei are mounted so that their anterior surfaces are directed toward the top of the plate.

FIG. 16. Interphase nucleus. Note a short and a long chromosomal sheath, each containing two chromosomal coils attached to the anterior margin of the nuclear membrane by an intranuclear chromosomal fibre. The posterior end of each sheath is attached to a portion of the chromatin nucleolus. × 3000.

FIG. 17. Prophase. The chromosomal sheaths are dividing longitudinally and each sheath contains a chromosomal coil attached anteriorly to the nuclear membrane and posteriorly to the chromatin nucleolus. The daughter chromosomes are coiled around each other. × 3000.

FIG. 18. Prophase. Daughter chromosomes arranged somewhat differently from preceding stage, the anterior attachments of the long pair having moved posteriorly, and each pair is attached posteriorly to a portion of the chromatin nucleolus. × 3000.

FIG. 19. Same as the two preceding stages except that each daughter chromosome is in the process of becoming attached to a single portion of the chromatin nucleolus. × 3000.

FIG. 20. Chromosomes have become short and stubby preparatory to poleward movement. × 4800.

FIG. 21. Early anaphase. The anterior ends of the long pair of chromosomes have made some progress in their poleward movement, one chromosome moving anteriorly, the other posteriorly. The short pair, as is sometimes the case, has been moved almost to the posterior end of the nucleus, although the chromosomes have not begun to separate. This was brought about by an extranuclear chromosomal fibre from the posterior pole (centriole) becoming attached to the intranuclear chromosomal fibre of the chromosome destined to go to the posterior pole. This fibre carried both chromosomes with it because the two chromosomes were connected. × 4800.

FIG. 22. A slightly later stage. The anterior ends of each pair of chromosomes have made considerable progress in their poleward movement, while the posterior ends are still connected and have not moved poleward. The short pair lies to the left and separation has progressed farther than in the pair which lies to the right. Note the evagination of the nuclear membrane at the four points where the intranuclear chromosomal fibres connect the chromosomes with the nuclear membrane. The evaginations are produced at these points by the pull of the extranuclear chromosomal fibres. × 3000.

FIG. 23. The chromosomes have turned completely around, a short one and a long one being directed toward each pole (anteriorly and posteriorly). Practically all of the connections between the daughter chromosomes have been broken. × 3000.

FIG. 24. The chromosomes have moved farther apart and the nucleus has begun to elongate. × 4800.

FIG. 25. The chromosomes have moved still farther apart and have begun to elongate. The nuclear membrane is constricting preparatory to being pulled in two. One intranuclear chromosomal fibre has been duplicated. × 3000.

FIG. 26. A later stage. Nucleus greatly elongated and will soon be pulled in two. Chromosomal coils in anterior pair are in the process of duplicating themselves by longitudinal division. × 3000.

FIG. 27. Posterior daughter telophase nucleus. The intranuclear chromosomal fibres and the chromosomal coils have been duplicated. The chromosomal sheaths, while slightly constricted, have not divided. × 4800.

PLATE 5

Spirotrichonympha polygyra

FIG. 28. Early stage in the separation of the four spiral flagellar bands into two groups. As the bands separate, they unwind. A centriole follows one band of each group from the anterior end to the posterior termination of the achromatic figure. Astral rays have arisen from the distal ends of each centriole; some of these rays have met and formed a portion of the central spindle; others, the posterior ones, are in the process of meeting to form more central spindle. × 1500.

FIG. 29. About the same stage as the previous one, but a different view of the two groups of separating and unwinding bands; anteriorly, the astral rays have met to form a portion of the central spindle; posteriorly, they have not met. × 1500.

FIG. 30. A slightly later stage in the separation of the bands and the formation of the central spindle portion of the achromatic figure. The astral rays have met both anteriorly and posteriorly to form the broad, flat central spindle. Note position of nucleus in this and in the two previous illustrations. × 1500.

FIG. 31. Later stage. Central spindle is longer, nearer the nucleus, bands are farther apart, and the unwinding of the bands has progressed considerably. × 1500.

FIG. 32. Still later stage. Central spindle is longer and lies over the nucleus. The bands which the centrioles follow are separated from the other bands. At the ends of the central spindle astral rays are arising from the centrioles and flagella are arising from the bands, but the flagella have been omitted and the astral rays have been omitted except those that joined to form the central spindle. × 1500.

FIG. 33. A much later stage. Nucleus has divided and daughter nuclei have moved a considerable distance apart; many of the astral rays have pulled apart so that the central spindle is now long and narrow. Note the manner in which the bands are unwinding. × 750.

FIG. 33 *A*. Detail of the anterior end of Fig. 33. Note the relation of the ends of the central spindle to the bands. × 1500.

PLATE 6

Spirotrichonympha polygyra

FIG. 34. Vertical view of a stage slightly later than Fig. 32. Note relation of central spindle to flagellar bands. The flagella are not drawn full length so as to avoid confusing them with the fibres of the central spindle. The astral rays that do not join to form the central spindle cannot be differentiated from flagella when the

two arise from the same point on the centrioles and flagellar bands. This is a six-banded individual. × 1500.

FIG. 35. An intermediate stage between Figs. 32 and 33 drawn to show that the ends of the central spindle are not flat but semicircular when the centrioles (and flagellar bands) from which the central spindle arises are not straight (as in Figs. 31, 32). In other words, the end of the central spindle has the same shape as the centriole (and band) from which the astral rays composing the central spindle arise. ×1500.

FIG. 36. A late stage in the unwinding of the flagellar bands. Achromatic figure has disappeared and daughter nuclei are far apart. New daughter axostyles are growing out and have extended a short distance beyond the nuclei posteriorly. × 1200.

FIG. 37. Elongate organism about to divide longitudinally. The unwinding of the parent bands is complete and new daughter bands will grow out presently. × 1200.

PLATE 7

Spirotrichonympha bispira

The flagella arising from the two parent or old bands and those arising from the new or free band are not drawn as long as they are. They are stopped where they leave the body.

Explanation of labels used in Plates 7–10.

a.e.—Anterior end (of new or free flagellar band).
a.r.—Astral rays.
a.r.f.c.—Astral rays from free centriole.
a.r.s.c.—Astral rays from stationary centriole.
c.—Centriole.
c.n.f.b.—Centriole of (or following) new flagellar band.
c.p.f.b.—Centriole of (or following) parent flagellar band.
c.s.—Central spindle.
e.e.—Elongating end (of new or free flagellar band).
f.—Flagella.
p.f.b.—Parent flagellar band.

FIG. 38. Anterior end of a cell in the earliest stage of division showing the two parent flagellar bands (*p.f.b.*), the new flagellar band and its centriole (*c.n.f.b.*), and flagella (*f.*), arising from the parent bands and the new band. The free centriole, which follows the new band from its point of origin in the anterior tip of the cell, has not begun to produce astral rays; nor has the stationary centriole which follows one of the parent bands. × 1500.

FIG. 39. A slightly later stage. The elongating end (*e.e.*) of the new band has begun to extend laterally, while the anterior end (*a.e.*) still remains at the anterior tip of the cell. × 1500.

FIG. 40. Later stage. The new band has begun to migrate posteriorly, its anterior end (*a.e.*) lying to the left and its elongating end (*e.e.*) to the right. The free centriole following the new band has given off astral rays which have joined some of those given off by the stationary centriole following one of the parent bands to form the very small, early central spindle (*c.s.*). In this instance, the centriole (*c.*) following one of the parent bands does not follow the band for its entire length, the distal portion being free. × 1500.

FIG. 41. The situation here is the same as in Fig. 40 except the new flagellar band is longer, has migrated farther posteriorly, the central spindle (*c.s.*) is longer and wider, and the stationary centriole is free of its band for a greater distance anteriorly. × 1500.

FIG. 42. Later stage. The new band, especially the elongating end (*e.e.*) has migrated farther posteriorly and now lies near the nucleus. The central spindle (*c.s.*) is longer. The distal portion of the stationary centriole (*c.*) is free of the band. × 1500.

FIG. 43. A considerably later stage in the development of the achromatic figure and the posterior migration of the new flagellar band. The central spindle (*c.s.*) is in two portions, one inside the parent flagellar bands and one outside. The portion inside the bands extends over the nucleus. No portion of the stationary centriole is free of the band. The shape of the end of the central spindle arising from the centriole following one of the parent bands is the same as the band and centriole from which it arises, semicircular. The other end of the central spindle is irregular in shape due to the position of the centriole from which it arises. Numerous astral rays (*a.r.*) extend toward the nucleus from the centriole that follows the new band. × 1500.

FIG. 44. Approximately the same stage as illustrated by Fig. 42, but different in appearance because of the position of the new band and its centriole. The end of the central spindle (*c.s.*) adjacent to the centriole of the new band is cylindrical or nearly so and no portion of the centriole of the parent flagellar band is free. × 1500.

FIG. 45. About the same stage as Fig. 44, but the achromatic figure presents a very different appearance because the new flagellar band and its centriole lie near the periphery of the cell and extend antero-posteriorly in almost a straight line, thus giving the posterior end of the central spindle a greatly flattened appearance. Note the length of the functioning portion of the centriole of the new band. × 1500.

FIG. 46. The achromatic figure here presents a strikingly different appearance from that of any of the previous illustrations, because many of the astral rays arising from the stationary centriole have extended posteriorly between the spirals of the parent flagellar bands instead of making their way toward the periphery, as a few of them have done, to join the astral rays arising from the free centriole (the one following the new flagellar band). Note astral rays extending from the free centriole toward the nucleus. When these rays join those arising from the other centriole and extending posteriorly within the spirals, another portion of the central spindle will be formed. This portion, like that illustrated in Fig. 43, will lie mostly within the flagellar bands. × 1500.

PLATE 8

Spirotrichonympha bispira

The flagella arising from the two parents or old bands and those arising from the new or free band are not drawn as long as they are. They are stopped where they leave the body. See explanation of Plate 7 for meaning of labels employed.

FIG. 47. The central spindle (*c.s.*) lies almost entirely outside the parent flagellar bands. Its posterior portion, which arises from the centriole of the new flagellar band, is broad and flat because the new band extends in almost a straight line, while its anterior portion, which arises from the centriole of one of the parent flagellar bands, is semicircular. × 1500.

FIG. 48. Here there are two portions of the central spindle (*c.s.*), one inside and one outside the spirals of the flagellar bands. The centriole (*c.*) following one of the parent flagellar bands is free of the band for a short distance. × 1500.

FIG. 49. The central spindle (*c.s.*) lies within the spirals of the flagellar bands except for the posterior portion which is posterior to the bands. Astral rays arise from both the stationary and free centriole and extend toward the nucleus. Some of these rays have joined the intranuclear chromosomal fibres in the nuclear membrane and by so doing have become extranuclear chromosomal fibres which are pulling the chromosomes toward the poles, the centriole of the parent flagellar band (*c.p.f.b.*) and the centriole of the new flagellar band (*c.n.f.b.*). × 1500.

PLATE 9

Spirotrichonympha bispira

The flagella arising from the two parent or old bands and those arising from the new or free band are not drawn as long as they are. They are stopped where they leave the body. See explanation of Plate 7 for meaning of labels employed.

FIG. 50. Note the manner in which the astral rays composing the anterior half of the central spindle arise from the distal end of the centriole following one of the parent flagellar bands. The central spindle is almost cylindrical at this end because of the spiraling of the portion of the centriole from which it arises, while the opposite end is almost flat. There are probably astral rays arising from the centriole of the new band and extending toward the nucleus but, since they cannot be distinguished with certainty from the flagella which are numerous in this area, they are not drawn. × 1500.

FIG. 51. This central spindle is almost cylindrical anteriorly, flat posteriorly, and twisted in the mid-region. The astral rays that have become extranuclear chromosomal fibres by attaching themselves to the intranuclear chromosomal fibres in the nuclear membrane, as well as the free astral rays, are intermingled with the fibres of the central spindle and can scarcely be distinguished from them, thus presenting a situation closely resembling that of many metazoan mitoses. × 1500.

FIG. 52. In this cell the new flagellar band and its centriole failed to migrate posteriorly so that the astral rays arising from the free centriole (*a.r.f.c.*) could not meet and join those arising from the stationary centriole (*a.r.s.c.*) to form a central spindle. × 1500.

PLATE 10

Spirotrichonympha bispira

The flagella arising from the two parent or old bands and those arising from the new or free band, except in Fig. 54, are not drawn as long as they are. They are stopped where they leave the body. See explanation of Plate 7 for meaning of labels employed.

FIG. 53. Another cell where the free band and its centriole failed to migrate posteriorly and the astral rays arising from the free centriole (*a.r.f.c.*) could not meet and join those arising from the stationary centriole (*a.r.s.c.*) to form a central spindle. This would be a telophase if the proper formation of the achromatic figure had not been prevented by the position of the centrioles, for the chromosomes are telophasic in structure. × 1500.

FIG. 54. Many of the astral rays arising from the stationary centriole have failed to meet those arising from the free centriole and only a small central spindle connects the two centrioles. The movement of the chromosomes and the division of the nucleus have been upset. The flagella of the new band are drawn full length, but only one flagellum is shown beyond the point of bifurcation. × 1500.

FIG. 55. Nucleus is being pulled in two and the elongate central spindle is pulling apart. × 1500.

FIG. 56. Nucleus has divided, achromatic figure has disappeared, chromosomes have elongated, new axostyles are developing, and the new or free flagellar band has migrated to the extreme posterior end of the parent cell. × 1500.

PLATE 11

Spirotrichonympha bispira

The flagella arising from the two parent or old bands and those arising from the first and second new bands are not shown as long as they are. They are stopped where they leave the body.

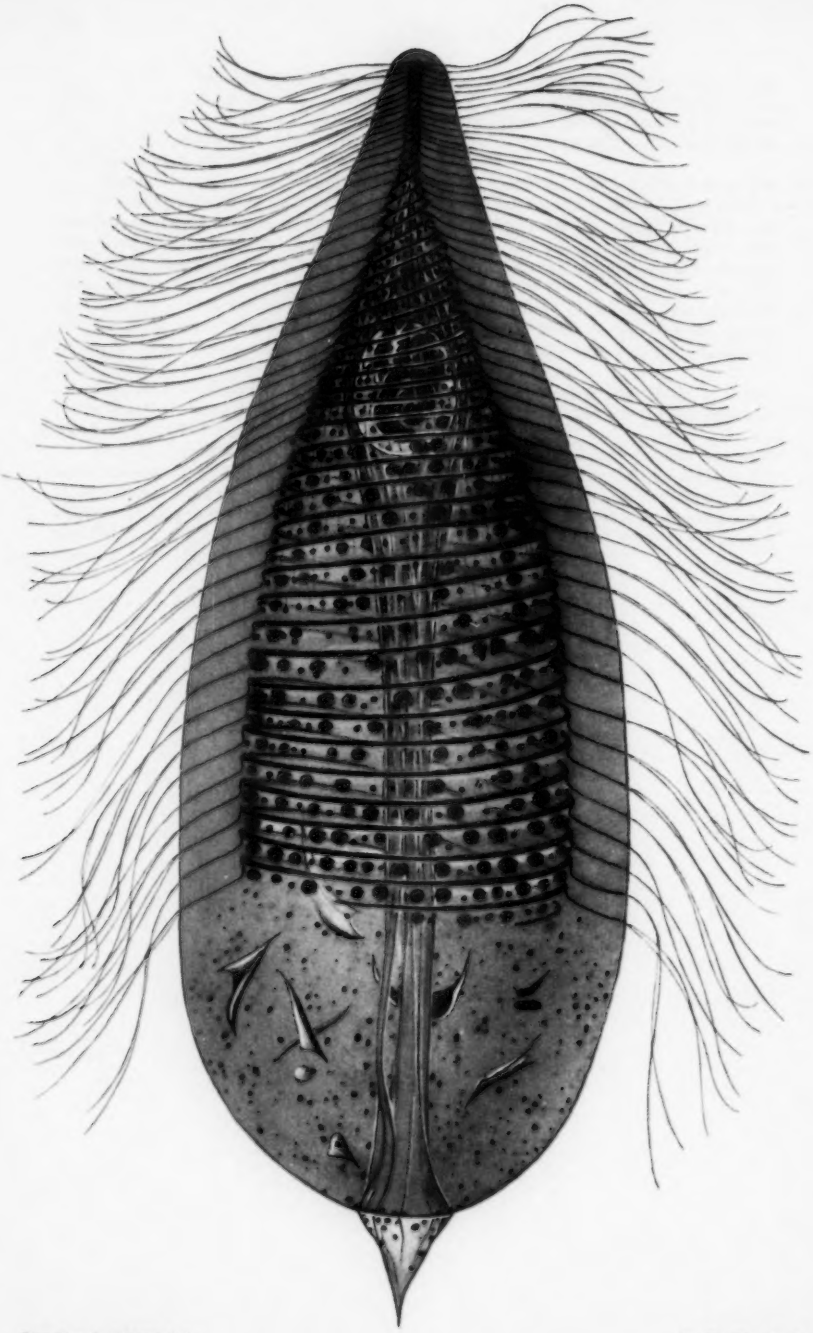
FIG. 57. The two parent flagellar bands, as in previous illustrations of cell division of *S. bispira*, remain intact. The new flagellar band which, although it has assumed various shapes in earlier stages, has not formed spirals, has now begun to spiral at its anterior end, which lies at the posterior end of the cell. The shape of the posterior end of the cell has changed, too. × 1500.

FIG. 58. Later stage. Cell is more elongate, posterior end is pointed more like the anterior end, and all of the new flagellar band has formed spirals. This stage is just before the transverse division of the cytoplasm to form two daughter cells; one, the anterior daughter, getting the two parent flagellar bands and their associated organelles; the other, the posterior daughter which is smaller, getting the new flagellar band and its associated organelles. Both daughters form new axostyles. × 1500.

FIG. 59. A posterior daughter cell after cytoplasmic division. The spirals of what was the new flagellar band before division of the body are little if any better arranged now. This organism has just separated from the anterior daughter. × 1500.

FIG. 60. A slightly later stage in the post-cytoplasmic development of a posterior daughter cell. The spirals of the flagellar band have perfected their arrangement. × 1500.

FIG. 61. A considerably later stage. A second new flagellar band has now appeared and is co-extensive with the first new band, the two forming spirals in the same manner as those of the parent cell previous to cell division. When these bands increase in length posteriorly (forming spirals as they do), the axostyle extends posteriorly, and the body increases in size, this cell will be like the parent which produced it. × 1500.



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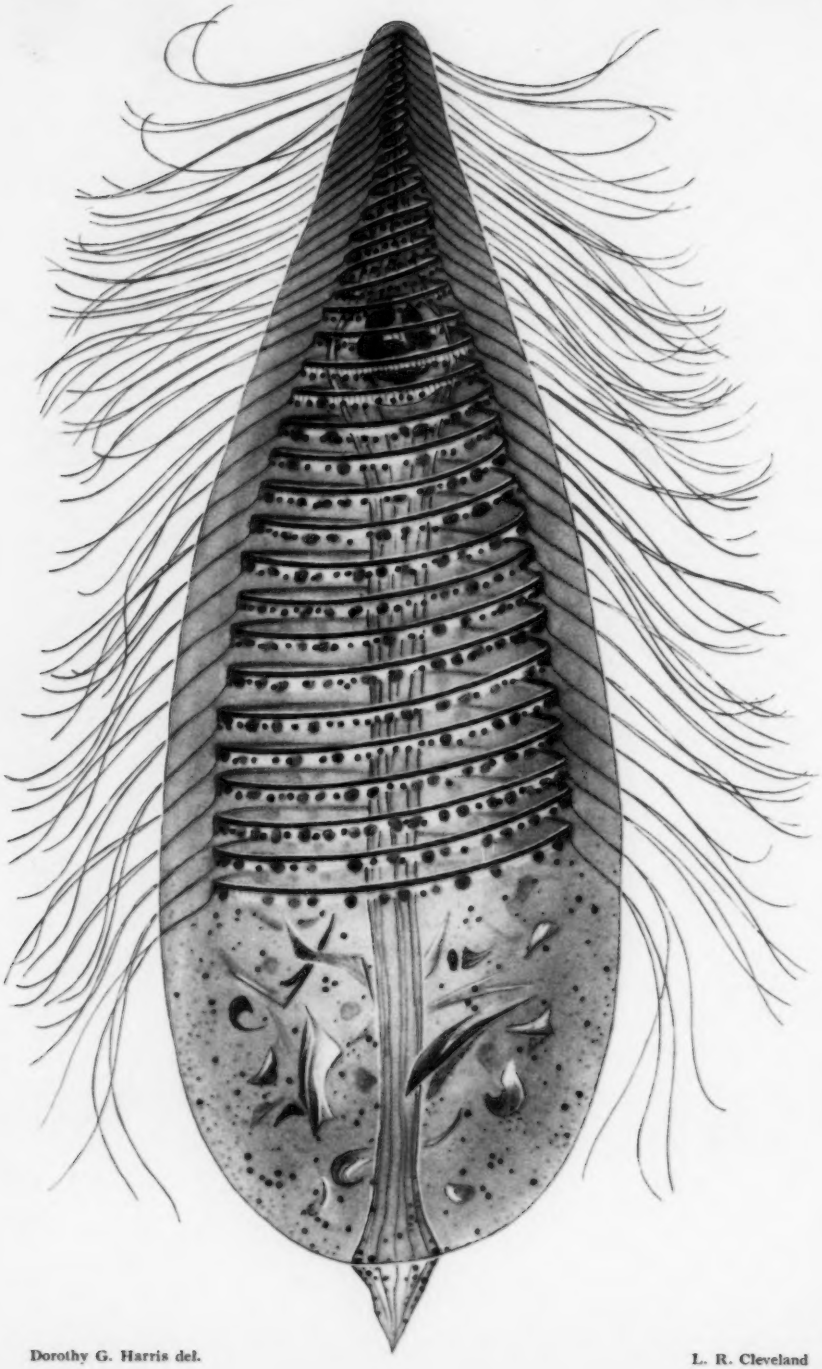

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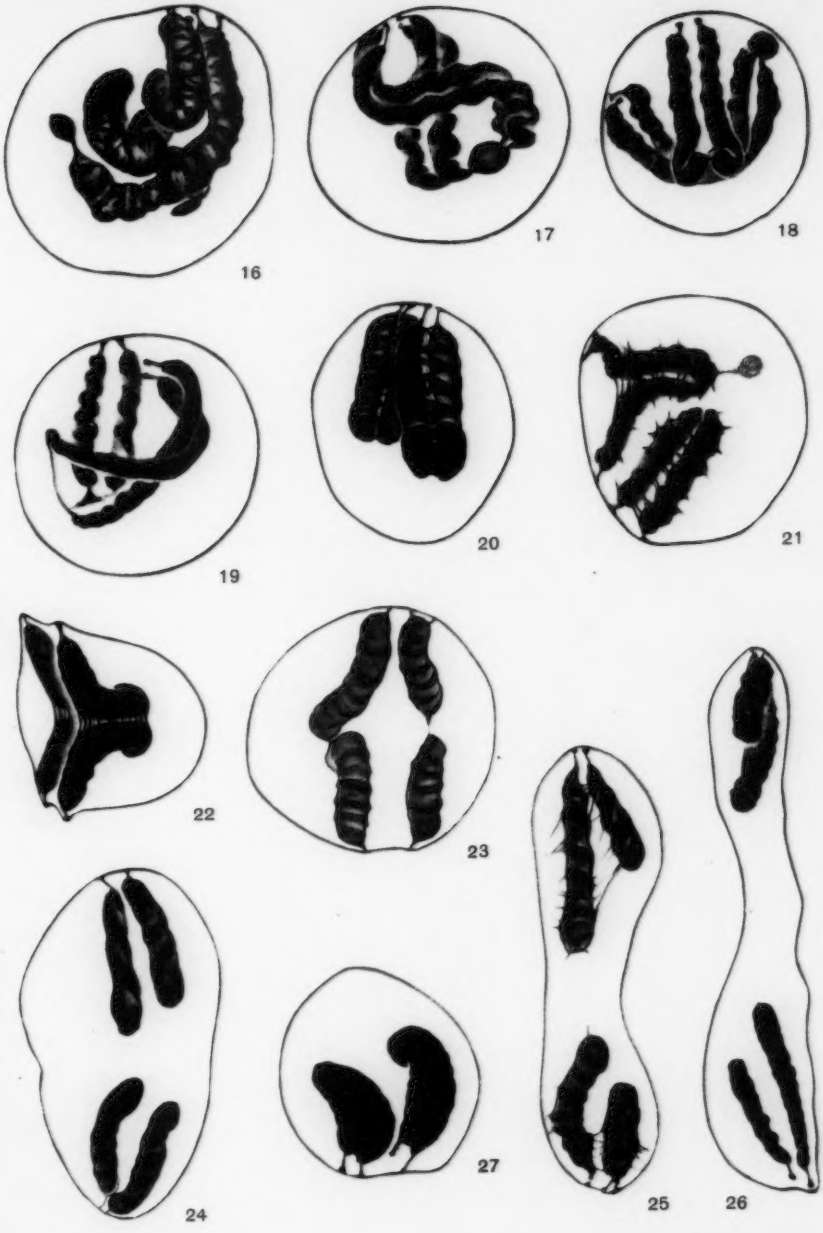

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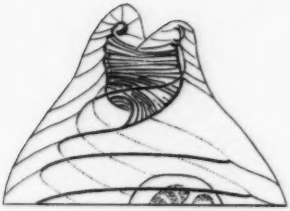

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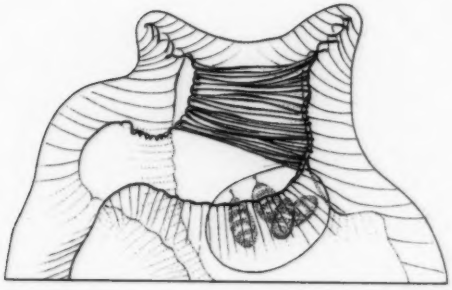

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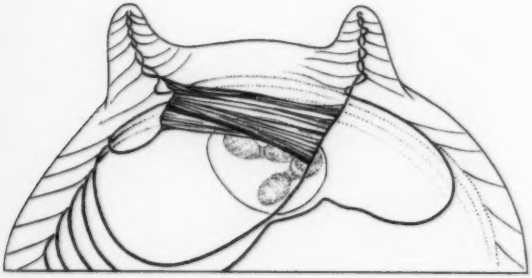

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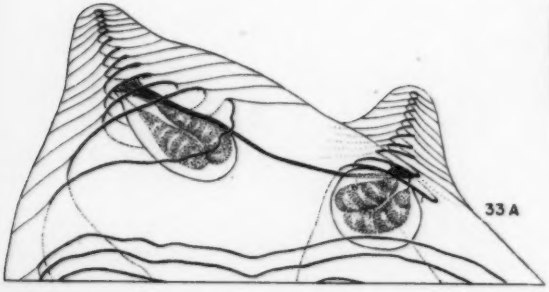

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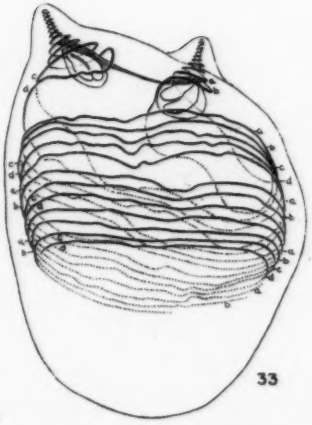

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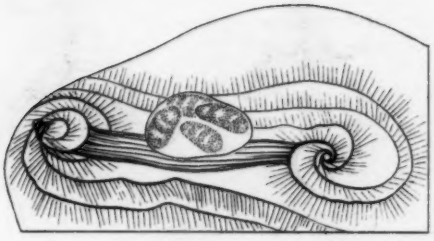

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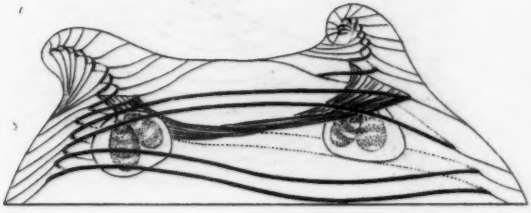

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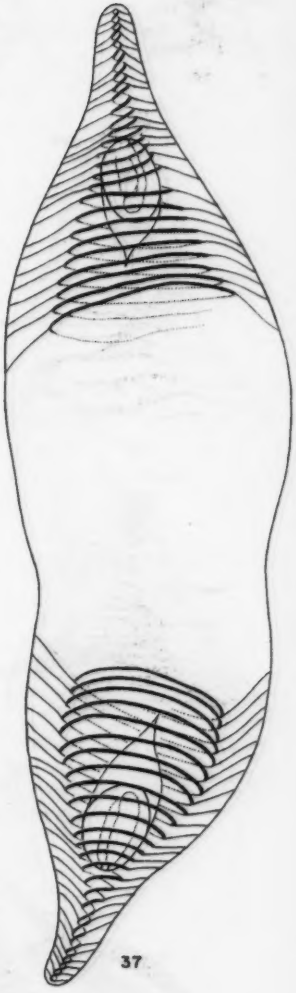

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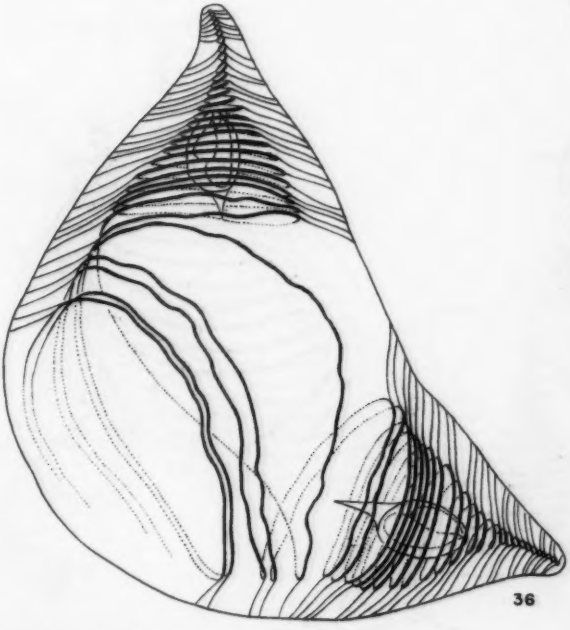

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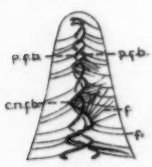

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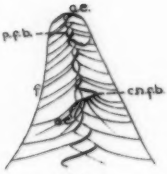

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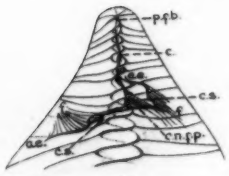

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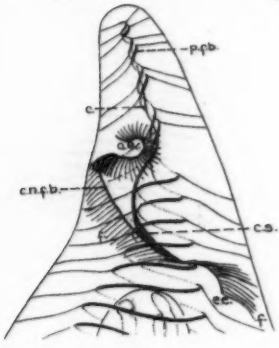

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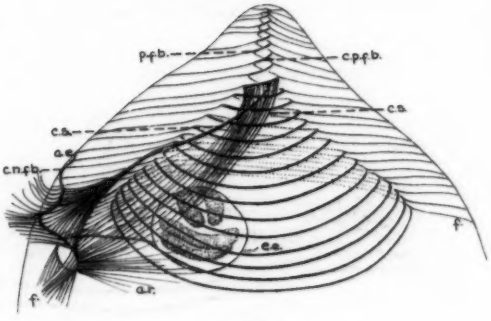

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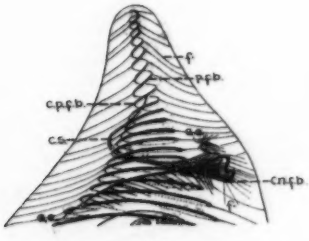

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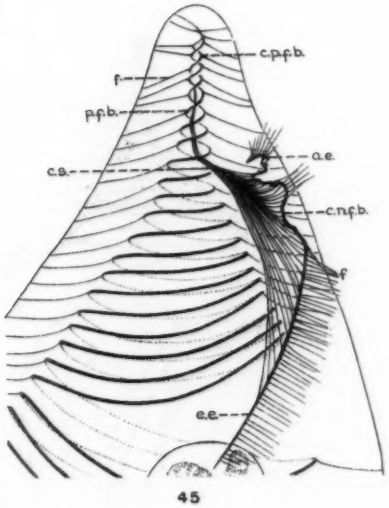

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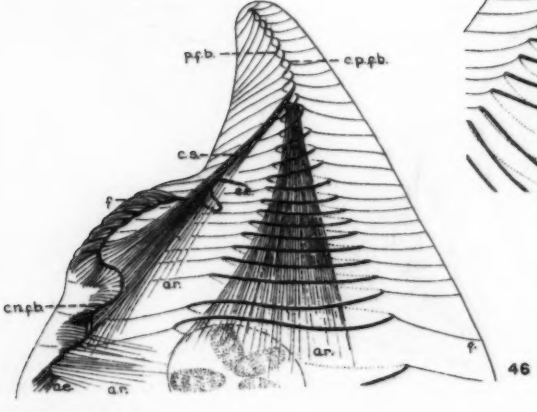

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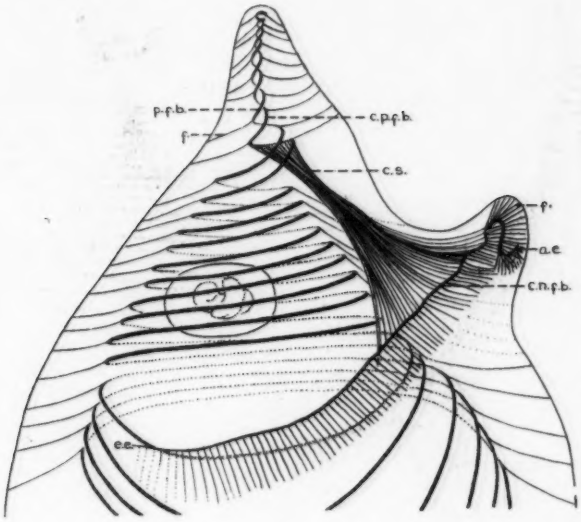

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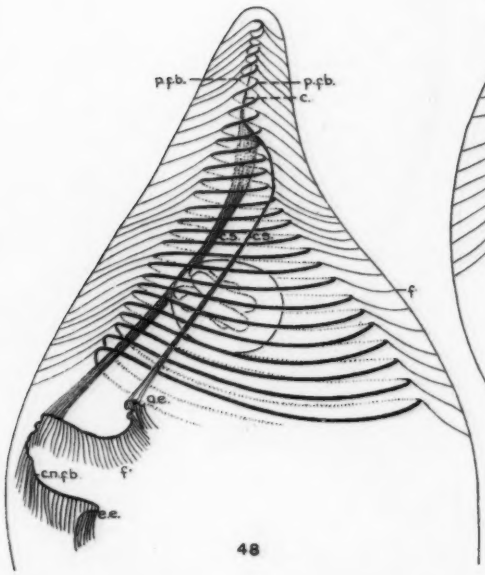

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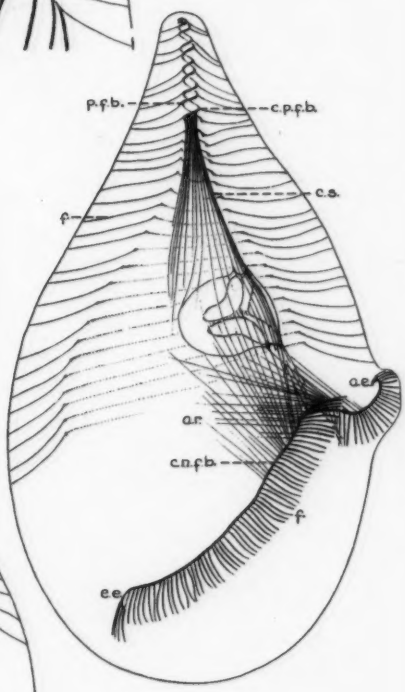

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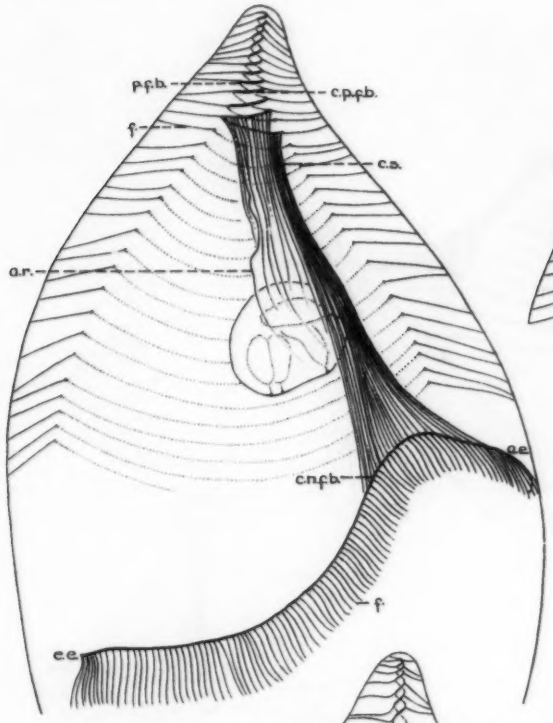

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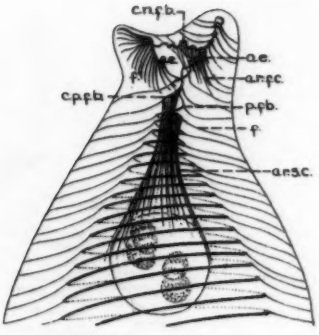

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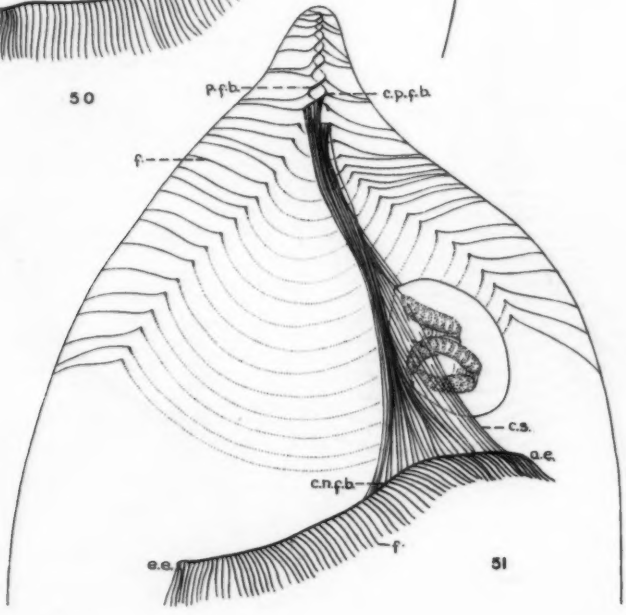

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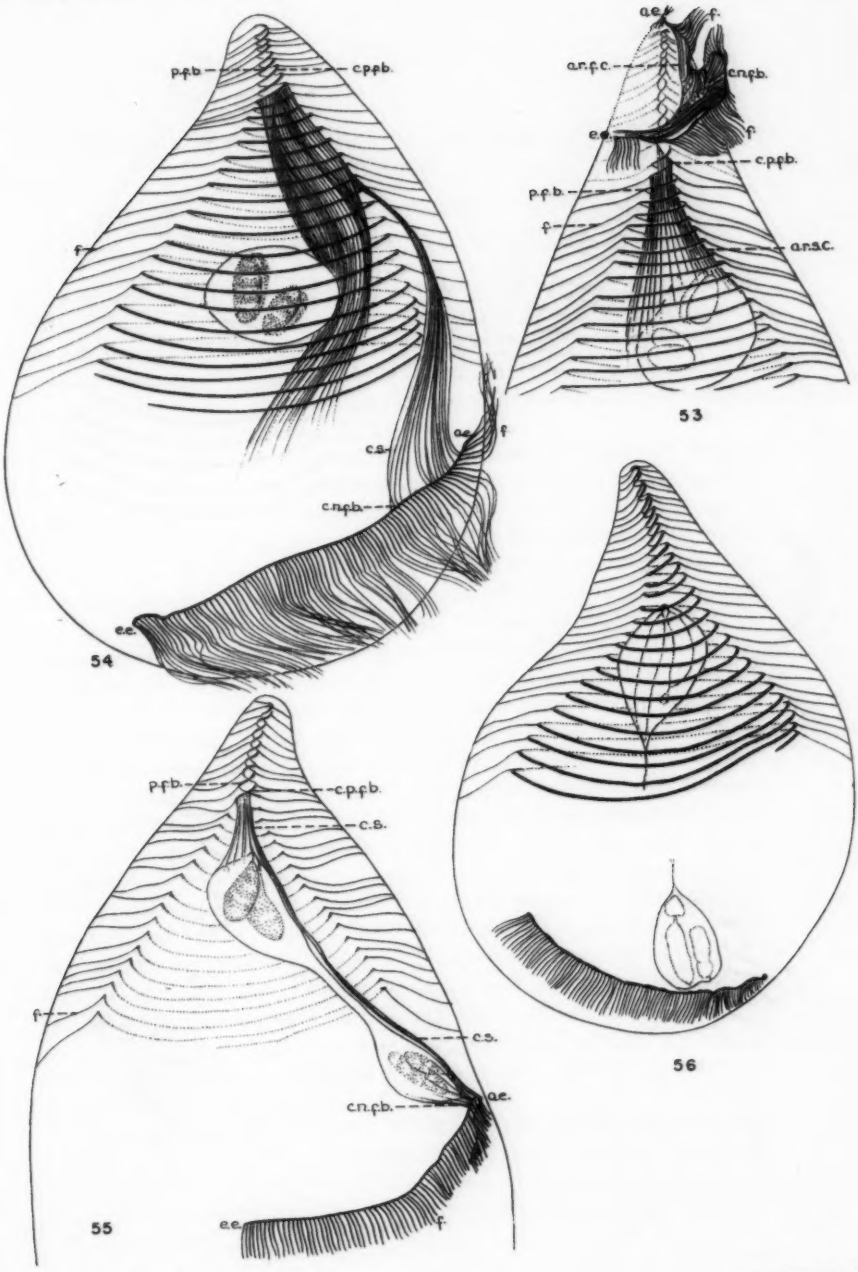

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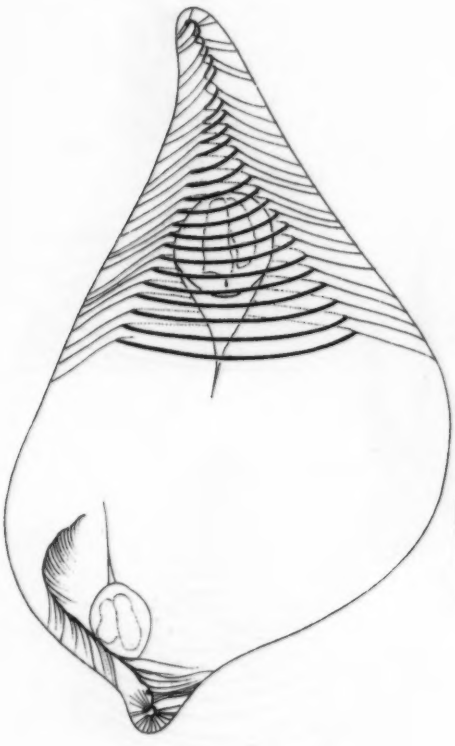

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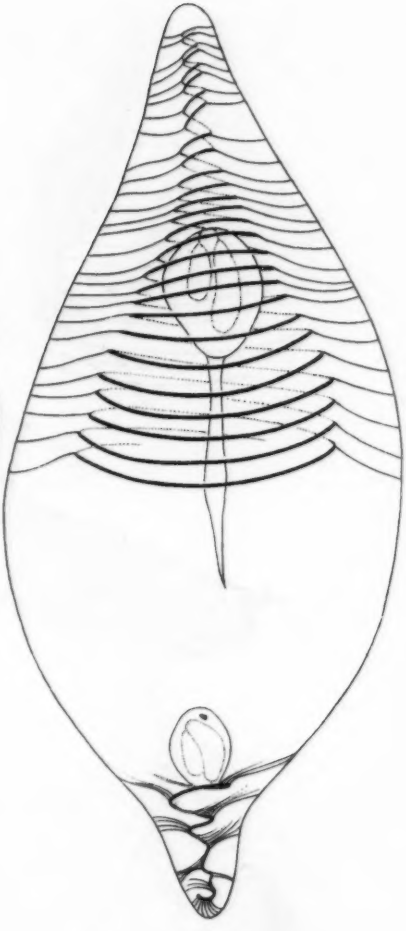

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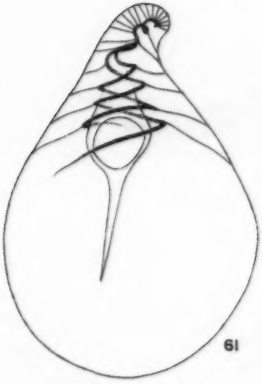

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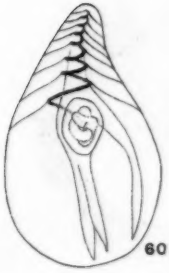

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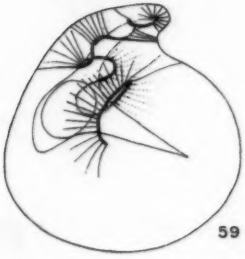

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ORIGIN AND DEVELOPMENT OF THE ACHROMATIC FIGURE

L. R. CLEVELAND

(*From the Biological Laboratories, Harvard University, Cambridge, Massachusetts*)

The question of the origin and nature of the achromatic figure has been debated for more than fifty years, and its existence has been seriously questioned many times, particularly during recent years. Several reasons have been advanced for regarding it as an artifact, the principal one being the inability to demonstrate clearly its origin, development, and function in living cells.

I have studied the achromatic figure in twenty genera of hypermastigote and ten genera of polymastigote flagellates. These organisms furnish a diversity of favorable material. In some of them, there is no question regarding the manner in which the achromatic figure is formed from the centrioles and the reality of the fibres composing it; in others, the development of the achromatic figure is not so plain, and can be determined with certainty only in view of their relation to those organisms where the process is unmistakably clear. There are gradations between the two groups. In several genera, the development of the achromatic figure has been followed in living cells from its very beginning to its disappearance in the late telophase; and the picture presented is exactly the same as in fixed cells.

In one genus, *Barbulanympha*, considerable time has been devoted to the origin and development of the achromatic figure when more than two centrioles are concerned in the process, and when only one is concerned; because the process is so very plain under these conditions that no one can question it. In a previous paper (Cleveland, Hall, Sanders, and Collier, 1934. *Mem. Am. Acad. Arts and Sci.*, **17**: 185) *Barbulanympha* was described, and an account was given of the manner in which the two elongate, interphase centrioles produce daughter or new centrioles like themselves, a process which need not be considered here; but it is desirable, before considering the achromatic figure when an abnormal number of centrioles are present, to consider it when two are present.

The centrioles vary in length in the four species of *Barbulanympha* from 15 to 30 microns; in the unstained living cell they are of a dense hyaline nature, and may be differentiated easily from other cellular contents; when fixed in Schaudinn's fluid and stained with Heiden-

hain's hæmatoxylin, they stain and destain in about the same manner as chromatin; they are joined at their anterior ends by a desmose; and their distal ends, which lie 20 to 30 microns apart, are free (Fig. 1).

The distal end of each centriole is surrounded by a spherical centrosome, which is 4 to 6 microns in diameter, and which moves with the centriole whenever the latter is moved, either by the natural movement of the cell or by mechanical manipulation. When the nucleus and centrioles are suitably destained for study, the centrocomes retain little or no stain.

In the interphase cell, there are no fibres extending from either centriole (Fig. 1). In the prophase, astral rays begin to grow out from the distal end of each centriole (Fig. 2). At first, the rays arising from one centriole are a considerable distance from those arising from the other; but, as they increase in length, the two sets of astral rays soon meet (Fig. 3), and, as they meet, the individual rays or fibres join, grow along one another and overlap to form the early central spindle portion of the achromatic figure (Fig. 4). That the astral rays arise from the centriole instead of the centrosome may be shown by observation of living cells; the rays may be traced through the centrosome to the centriole. The same may be demonstrated also in fixed cells. And further proof that the centrosome plays no part in the production of the astral rays may be obtained from a study of several other genera with elongate centrioles similar to those of *Barbulanympha* but with no centrosomes surrounding their distal ends; in these, the connection of the astral rays to the centrioles may be seen at a glance. Those genera with centrosomes surrounding the distal ends of their centrioles form cylindrical central spindles, while those with no centrosomes form flat ones; and thus the rôle of the centrosome in directing the astral rays that form the central spindle may be seen.

As the astral rays continue to increase in length, more of those arising from one centriole meet those arising from the other, and the central spindle becomes larger; and, at the same time, the central spindle increases in length, because the centrioles from which it arises move in opposite directions (Fig. 5). Those astral rays which do not participate in the formation of the central spindle, because they do not meet and overlap, also increase in length; at the same time, more astral rays arise from each centriole, so that the individual rays vary greatly in length; and it is not long after the central spindle is formed before some of the astral rays which do not participate in its formation become extranuclear chromosomal fibres by joining the intranuclear chromosomal fibres, the fibres which anchor the chromosomes to the ever intact nuclear membrane (Fig. 6). Thus, the achromatic figure

consists of astral rays which are joined together to form the central spindle, astral rays which are connected to the chromosomes and are responsible for their movement to the poles, and astral rays which merely radiate in the cytoplasm and, so far as known, perform no function. All of these fibres appear alike structurally, and the only justification for naming them is their functional differences.

When three centrioles are present in the interphase (Fig. 7), the type of achromatic figure produced by them during cell division varies considerably, depending on their position. If the distal ends of two of the centrioles lie fairly close together, as illustrated in Fig. 8, two long central spindles and one short one are produced. In this instance, the centriole which lies somewhat apart functions jointly with each of the other centrioles in the production of the two long central spindles; and the two centrioles which lie fairly close together function jointly in the production of a short central spindle between them. In other words, the astral rays arising from the centriole which lies more or less alone, and which may be termed the remote centriole, meet, join, and overlap those arising from the two centrioles which lie fairly close together, and thus the remote centriole functions as much again as each of the other centrioles in the formation of the two long central spindles, because it supplies as many astral rays for central spindle production as both of the other centrioles combined. If, however, as is frequently seen, two centrioles lie adjacent and one apart, the three centrioles function in the production of one central spindle, which has the same appearance as when only two centrioles are functioning in the production of a central spindle (for example, as in Fig. 5). The achromatic figure appears exactly as that of Fig. 8 would appear if the two centrioles on the right were pulled together so that one centrosome touched the other.

If the distal ends of the three centrioles are more or less equidistant, i.e., at the apices of an equilateral triangle, each centriole functions in approximately the same manner in the production of the achromatic figure; three central spindles, more or less equal in size, are produced, and each centriole functions in conjunction with two other centrioles in their formation (Fig. 10). If, on the other hand, the three centrioles are not equidistant, but lie at the apices of a triangle which is wider on one side, only two central spindles are produced—at least at first; a third may be formed later, as the astral rays become longer, if the centrioles between which a central spindle failed to form earlier do not lie too far apart (Fig. 9). Three is the maximum number of central spindles that can be produced from three centrioles.

Many cells with four centrioles have been studied. The four inter-

phase centrioles may all be mature, i.e., fully grown, or some of them may be in the process of development, as shown in Fig. 11; but they do not function until growth is completed; and when they function, the type of achromatic figure produced by them depends on their position. If they lie side by side in groups of two, one central spindle is produced, although all four centrioles participate in its formation; if they lie at the four corners of a square, six central spindles are produced; if they lie at the corners of a rectangle, four central spindles are usually produced; if they lie fairly close together, but not adjacent, in groups of two, two central spindles are usually produced in the early stages of cell division, and in the later stages, if the centrioles of each group move apart, a central spindle is formed between the centrioles of each group. Sometimes, the four centrioles lie in the same plane in groups of two in such a manner that a criss-crossing occurs in the astral rays forming the central spindles. Figure 13 illustrates an example of this. Here astral rays arising from the upper centriole on the right have joined astral rays arising from both of the centrioles on the left, so that this centriole is connected by a central spindle to each of the centrioles on the left; and the astral rays arising from the lower centriole on the right have joined astral rays arising from both of the centrioles on the left, so that this centriole is also connected by a central spindle to each of the centrioles on the left. In brief, each of the four centrioles is connected to two other centrioles by a central spindle. Would such a series of connections occur as a result of "lines of force"? Other instances have been seen where, for example, the lower centriole on the right was connected to each centriole on the left by a central spindle, while the upper centriole on the right was connected only to the upper centriole on the left by a central spindle. And other variations have been seen in the criss-cross central spindle interconnections of centrioles.

When five non-adjacent centrioles are present, eight central spindles could be formed by an overlapping of the astral rays arising from them, although six, as shown in Fig. 12, is the usual number. Here each of the two upper centrioles is functioning in the formation of three central spindles; the lower right and left centrioles are each functioning in the formation of two central spindles; and the lower middle centriole is functioning in the formation of four central spindles, most of its astral rays being used to form central spindles.

Many examples of a still larger number of centrioles functioning in the production of multiple achromatic figures have been studied, but only one has been illustrated; this is Fig. 16 where fifteen centrioles are present (in order to avoid confusion only the centrosomes are drawn).

Here there is great variation in the length and width of the central spindles, resulting from differences in the time of their formation and in the number of fibres composing them. There are also many instances of the criss-crossing of the fibres of the central spindles; instead of the astral rays arising from one centriole joining those from another, they join those from several centrioles. Few of the central spindles are straight, that is, extend directly from one centriole to another; and it is difficult, owing to their curvation and the manner in which they are crowded together, to determine the number present.

When two centrioles are present but are so widely separated that the astral rays arising from one cannot meet those arising from the other, the central spindle portion of the achromatic figure is not formed (Fig. 14). Similarly, when only one centriole is present, the development of the achromatic figure is incomplete; many astral rays extend to and around the nucleus, but they do not form a central spindle (Fig. 15).

In a few instances where several interphase centrioles are present, decidedly abnormal conditions have been seen; the formation of centrosomes surrounding the distal ends of the centrioles is either completely or partially upset, and strands, varying in size from a little larger than astral rays to half the size of the centrioles, extend posteriorly from the centrioles (Fig. 17). Just what these strands are is not known. They can scarcely be regarded as astral rays, or even a premature aberrant attempt to produce an achromatic figure; but they may be secondary extensions from the centrioles.

I am indebted to the Penrose Fund of the American Philosophical Society for financial assistance.

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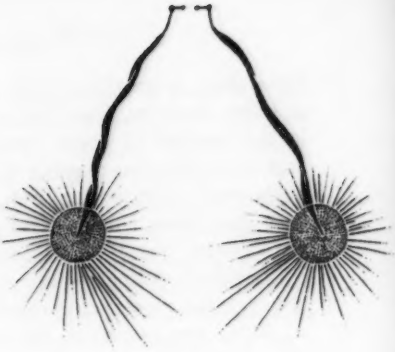

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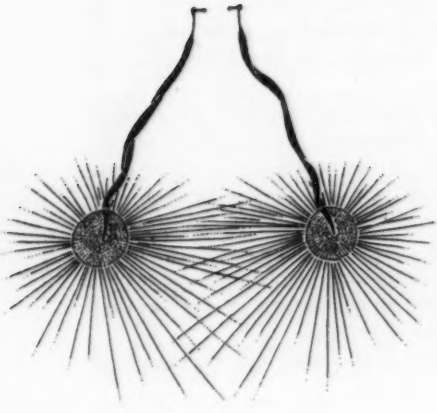

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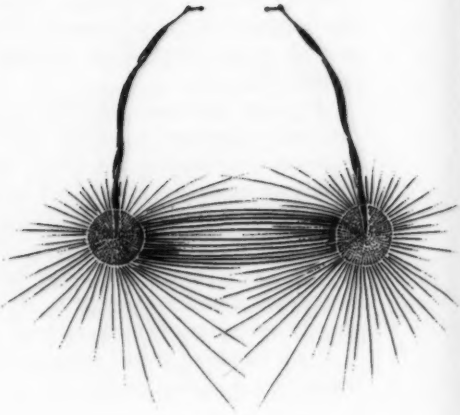

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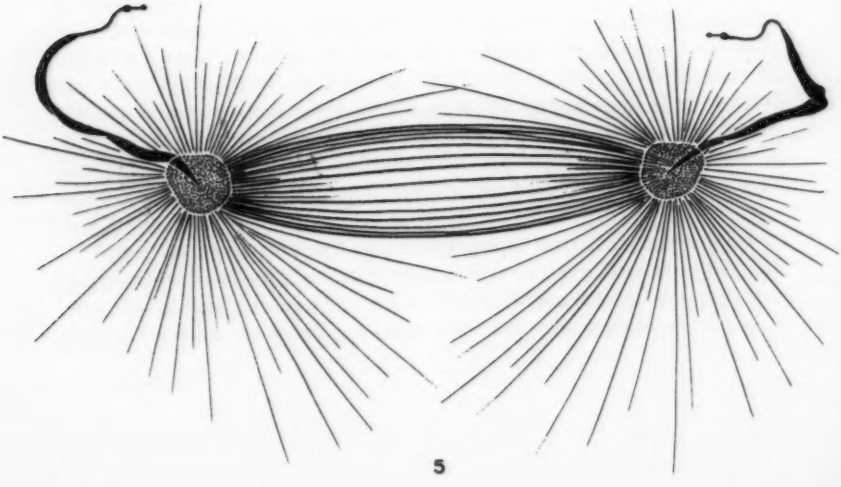

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EXPLANATION OF PLATES

All figures were drawn with the aid of a camera lucida from material fixed in Schaudinn's fluid and stained with Heidenhain's hæmatoxylin. The organism is *Barbulanympha*.

PLATE 1

FIG. 1. The two elongate interphase centrioles with centrosomes surrounding their distal ends. × 1400.

FIG. 2. Prophase centrioles with astral rays arising from their distal ends. × 1400.

FIG. 3. The astral rays arising from one centriole are meeting those arising from the other, preparatory to the formation of the central spindle. × 1400.

FIG. 4. The astral rays have met, joined, and grown along one another to form the early central spindle. × 1400.

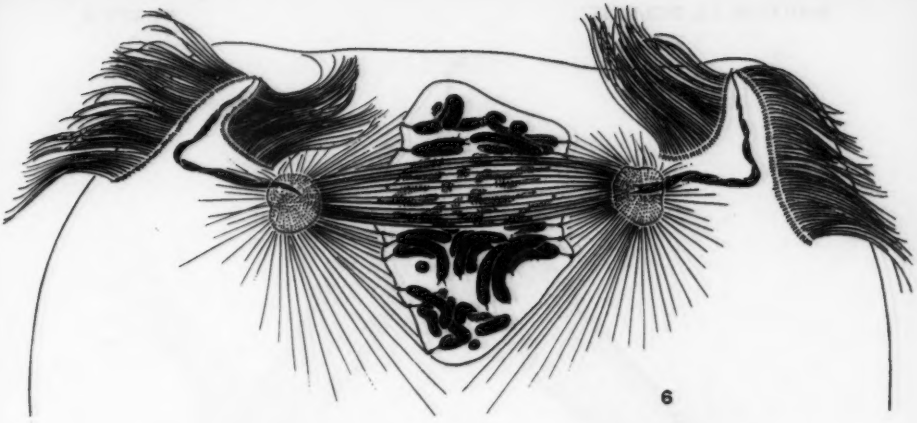
FIG. 5. More astral rays have joined and the formation of the central spindle is complete. × 1400.

PLATE 2

FIG. 6. Longitudinal section of anterior end of cell showing the interrelation of flagellated areas, centrioles, achromatic figure, and chromosomes. Some of the astral rays which do not join and overlap in the formation of the central spindle have become extranuclear chromosomal fibres by connecting with the intranuclear chromosomal fibres in the nuclear membrane. × 1400.

FIG. 7. Three interphase centrioles and centrosomes. × 1400.

FIG. 8. The type of achromatic figure produced by three centrioles when they lie in this position. × 1400.



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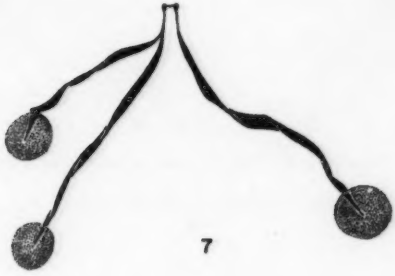

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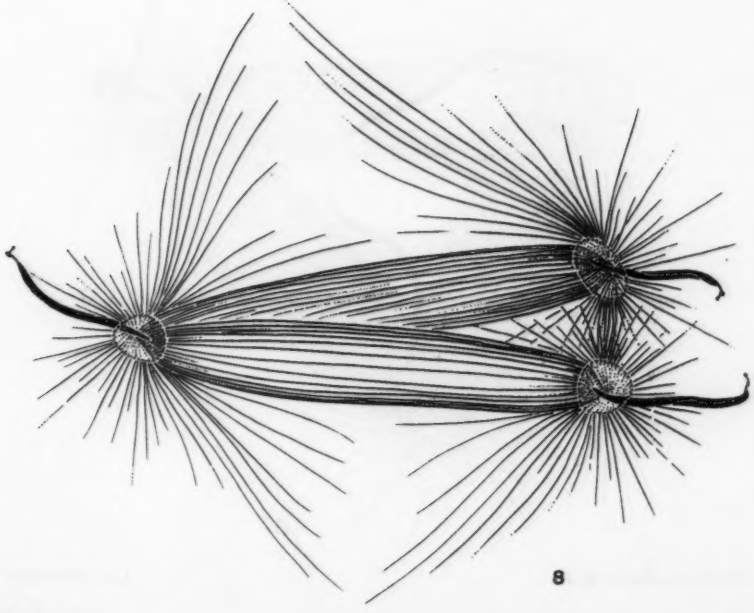

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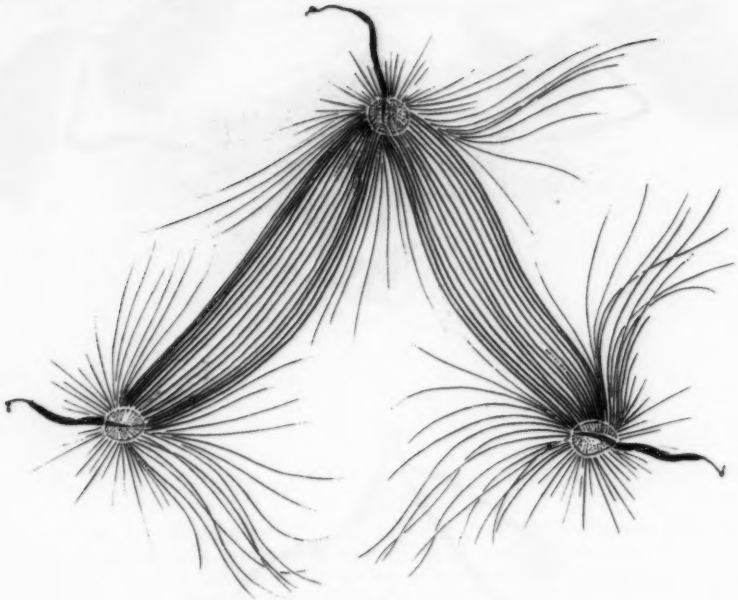

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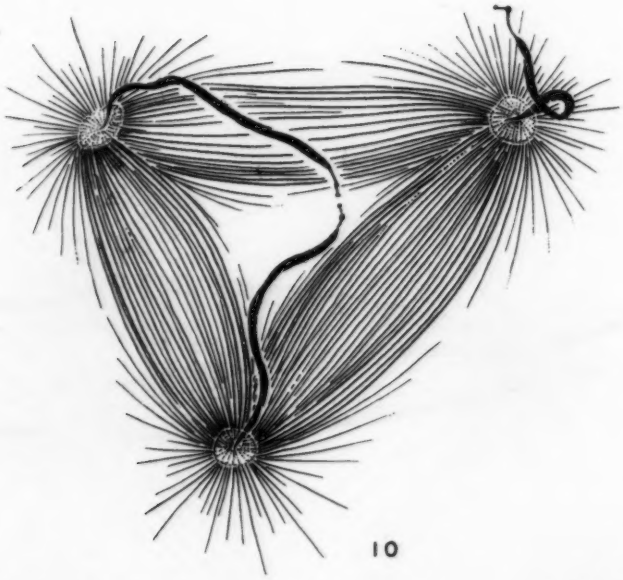

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PLATE 3

FIG. 9. Three centrioles in another position and the achromatic figure produced by them. × 1600.

FIG. 10. Still another position of three centrioles and the achromatic figure produced by them. × 1600.

PLATE 4

FIG. 11. Four interphase centrioles, one mature and three immature. × 1400.

FIG. 12. Five centrioles and their achromatic figure. × 1400.

FIG. 13. Four centrioles and their achromatic figure. Note the criss-crossing of the astral rays forming the central spindle. × 1400.

FIG. 14. Two centrioles so far apart that their astral rays could not meet to form the central spindle portion of the achromatic figure. × 600.

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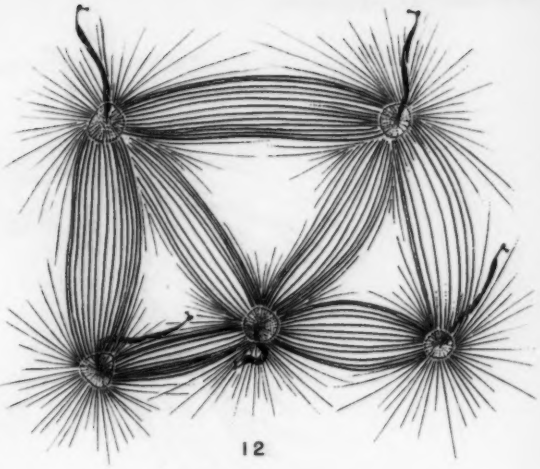

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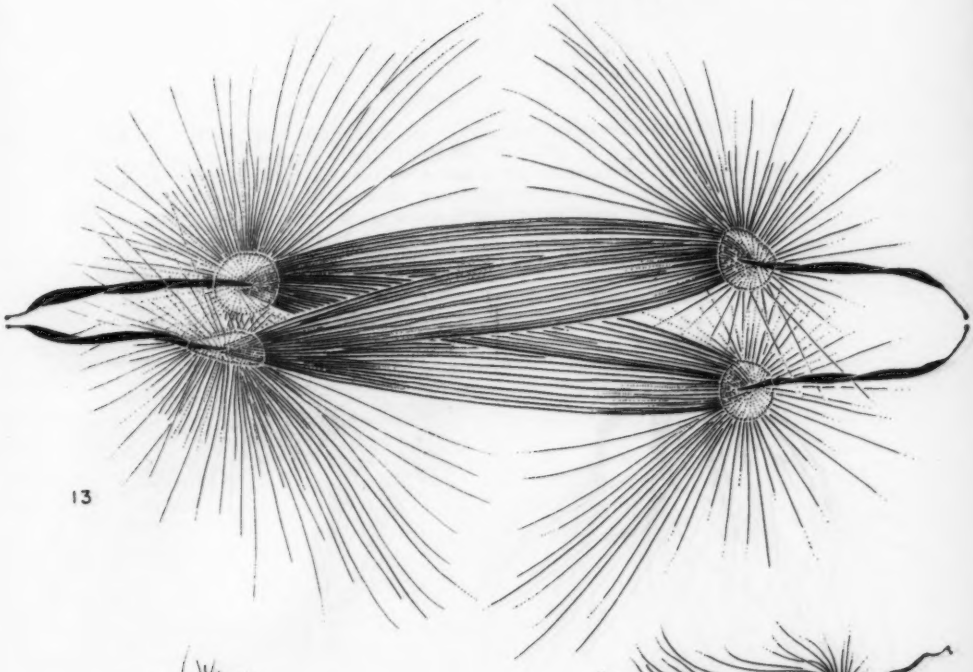

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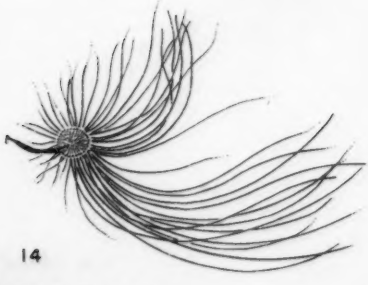

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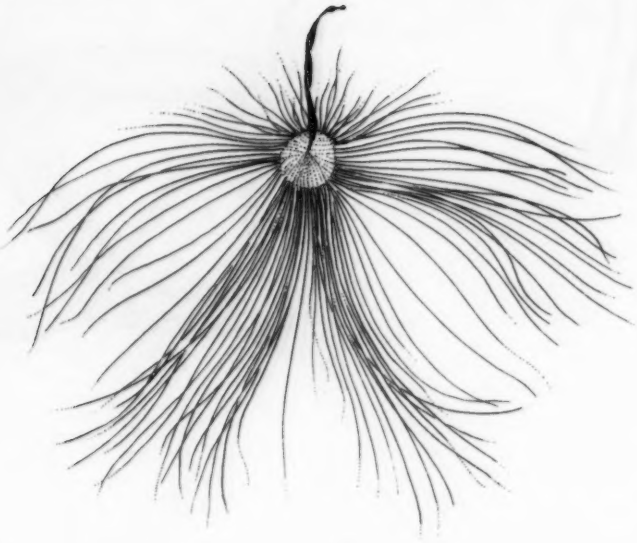

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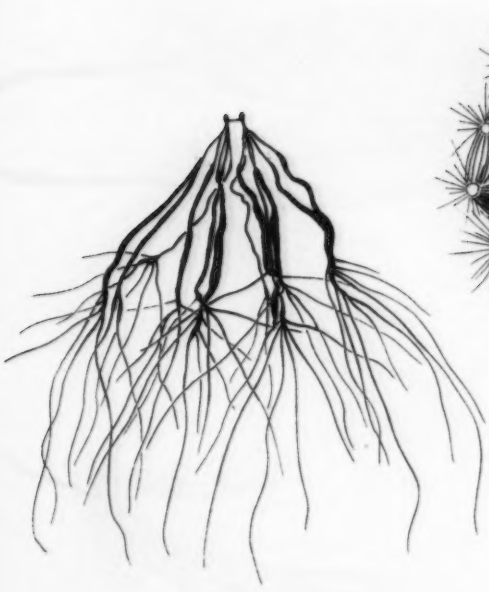

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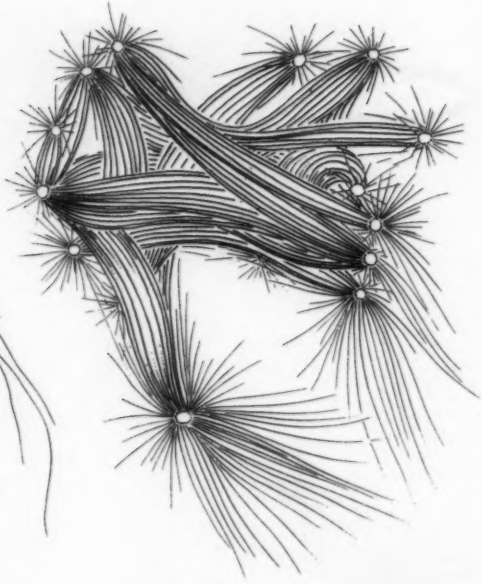

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PLATE 5

FIG. 15. The type of achromatic figure produced when only one centriole is present. Note the absence of a central spindle. × 1400.

FIG. 16. Fifteen centrioles are functioning in the production of an achromatic figure. In order to avoid confusion, the centrioles are omitted; but the position of their distal ends is indicated by the position of the centrosomes. × 1400.

FIG. 17. Interphase centrioles with probable secondary posterior extensions. × 1400.

THE ADSORPTION OF BACTERIA BY MARINE BOTTOM [1]

SELMAN A. WAKSMAN AND UNTO VARTIOVAARA

(From the Woods Hole Oceanographic Institution and New Jersey Agricultural Experiment Station)

In various recent studies (1, 4, 5) on the adsorption of bacteria in soil, the impression is left that the bacteria exert, in that state, only little effect upon the cycle of life in the soil. In his book on Agricultural Microbiology, Chudiakov (2) states emphatically that the problem of the condition of the bacteria in the soil hampered considerably a better understanding of soil microbiological processes. The existence of a surface relationship between the soil and microörganisms is true not only of bacteria but also of Protozoa, as shown by Cutler (3).

Different species of bacteria were found to behave differently in regard to their adsorption by the soil particles; their mobility and ability to form zoöglea seemed to be of special significance in this connection. The chemical activities of the bacteria, as measured by the evolution of CO_2, were considerably modified by the adsorption process (4). The finer soil constituents, namely the clay and silt fractions, were found to have a much greater adsorptive effect than the sand. Different soil types were found to adsorb bacteria to a different degree (5).

Rubentschik (8) made a detailed study of the phenomenon of bacterial adsorption in salt basins; the bacteria isolated from the mud were found to show a higher degree of adsorption than the bacteria found in the water. The conclusion was reached that the bacterial benthos consist of easily adsorbable species whereas the bacterial plankton contains organisms possessing a low degree of adsorption by the bottom sediments. A bacterial exchange took place in the mud, the adsorption of some species being accompanied by the desorption of others from the mud. The relative concentration of the bacteria, their nature and the type of bottom material exerted an influence upon the adsorption process. The activities of different species of bacteria were variously modified in the adsorbed state: the metabolism of some was lowered and that of others was increased.

[1] Contribution No. 161 of the Woods Hole Oceanographic Institution and Journal Series Paper of the New Jersey Agricultural Experiment Station, Department of Soil Microbiology.

According to Peele (6), the adsorption of bacteria is probably due to the attraction of unlike electric charges. The nature of the base in the soil complex influences considerably the process of adsorption, the monovalent cations showing the least adsorption.

In most of these studies, pure cultures of bacteria were used and the results obtained interpreted in terms of processes carried out by a complex population of microörganisms inhabiting the soil or the sea bottom. Usually a very short period of contact was allowed between the soil and the bacterial culture grown upon an artificial medium; no attempt was made to determine what happened after the culture had adjusted itself to the environment.

These studies have an important bearing upon the influence of the sea bottom upon the bacterial activities in the water. A number of questions may arise in this connection; 1. Is the specific occurrence of certain bacteria in the bottom due to their adsorption by the bottom material? 2. Is the relatively low number of bacteria in the sea water, as compared with that in the bottom, due to their removal from the water by the adsorption process? 3. Is this relationship responsible for the difference in the rate of bacterial processes taking place in shallow seas over a sand bottom such as Georges Bank, compared with the corresponding processes over a mud bottom, such as the Gulf of Maine (7, 11)?

These and other questions deserve fundamental treatment. No attempt will be made to give a definite answer in the following experiments, which must be considered as preliminary in nature.

Experimental

In order to eliminate the interfering effect of the bacterial population normally found in fresh bottom material, the first experiments were carried out with marine mud and sand which had been kept in a dry state for 5 years and in which the bacteria had been reduced to very low numbers.

In the first experiment, the adsorption of bacteria from a mixed population and from a pure culture of a marine bacterium by two different types of dry marine mud and sand was studied. A mixed culture was obtained by allowing fresh sea water to remain, in a glass container, in the laboratory, for a period of 48 hours. This resulted in an increase in the numbers of bacteria from a few hundred to 303,000 per 1 cc., as determined by the plate method. As a pure culture, a marine agar liquefying bacterium (No. 11) was used. This organism was selected because of the ease of recognizing the colonies produced on the plate. It had been isolated from sea water and kept in culture for a period

of 2 years. The organism was grown in a medium poor in nutrients (1 gram peptone, 1 gram glucose and 0.5 gram K_2HPO_4 in one liter of sea water), for 24 or 48 hours. The culture was then diluted ten times with sea water sterilized by heating 30 minutes at 80° C. The two muds, No. 1329 and No. 1331, contained 2.46 and 1.58 per cent organic carbon and 0.28 and 0.16 per cent nitrogen, respectively (9). The sand contained 0.58 per cent carbon and 0.05 per cent nitrogen. Ten-gram portions of mud or sand were placed in 250-cc. flasks containing either 100 cc. cultured sea water or 100 cc. of the diluted 24-hour culture of the bacterium. The flasks were shaken by hand for 10 minutes, allowed to stand 10 minutes, and 1-cc. portions of the supernatant liquid plated out using a sea water agar medium (No. 1). The

TABLE I

Adsorption of bacteria from mixed and pure cultures by dry marine mud and sand

Nature of culture	Bacteria in 1 cc. water, thousands			
	Start	*10 minutes*	*2 hours*	*21 hours*
Cultured water				
Control	303	—	—	—
Plus mud 1329	—	28	13	3,700
Plus mud 1331	—	25	75	530
Plus sand	—	180	115	32,000
Bacterium No. 11				
Control	1,600	—	—	—
Plus mud 1329	—	9,620	6	5
Plus mud 1331	—	13,000	21	365
Plus sand	—	22,000	40,000	40,000

flasks were kept in the laboratory, with occasional shaking, and water plated out again after 2 and 21 hours.

The results presented in Table I show that the pure culture of the bacterium was readily adsorbed by the marine mud. The sand, however, had no adsorptive effect; there was, in fact, a marked increase in bacterial numbers due to the separation of the bacterial masses or clumps into individual cells as a result of shaking with the sand. In the case of the crude cultures (i.e. the bacterial population in the cultured sea water), the immediate adsorption of the bacteria was followed by a rapid increase in numbers. The latter was due to the rapid multiplication of certain bacterial forms at the expense of the organic matter in the mud and sand.

This experiment was repeated and an attempt made to measure not only the multiplication of the bacteria, but also their activities, as influenced by the presence of bottom material. This could best be

done by determining the oxygen absorption, as a result of bacterial multiplication (10). Two-gram portions of the two muds and 5-gram portions of the sand were placed in oxygen bottles of about 220 cc. capacity. The bottles were filled with 3-day-old cultured sea water. They were closed and incubated at room temperature, under water and in the dark. At different intervals, some of the bottles were removed and analyzed for bacteria, by plating out 1 cc. of the supernatant water, and for oxygen by the Winkler method.

The results reported in Table II show that there was comparatively little adsorption of bacteria from the mixed bacterial population in cultured sea water, by either marine mud or sand. On the contrary, there was a marked rise in bacterial numbers, reaching a peak in 17 to

TABLE II

Influence of marine bottom material on the growth and metabolic activity of a bacterial sea water population

Nature of culture	Bacteria in 1 cc. water, thousands					
	Start	*3 hours*	*17 hours*	*42 hours*	*4 days*	*8 days*
Sea water control	0.5	0.9	1.2	1.0	7.3	3.1
Plus mud 1329	1.8	1.9	1,175.0	850.0	133.0	12.2
Plus mud 1331	1.9	1.7	1,240.0	1,435.0	205.0	3.3
Plus sand	2.2	3.9	1,255.0	615.0	65.0	3.3
	Oxygen consumed, cc. per liter					
	Start	*3 hours*	*17 hours*	*42 hours*	*4 days*	*8 days*
Sea water control	0.0	0.03	0.07	0.23	0.38	0.64
Plus mud 1329	0.0	0.25	0.73	1.42	2.53	3.48
Plus mud 1331	0.0	0.13	0.62	1.29	2.47	3.31
Plus sand	0.0	0.19	0.88	1.57	2.18	3.24

42 hours, followed by a rapid decline. Active oxygen absorption took place in the bottles accompanying bacterial multiplication; this was due to the oxidation of the organic matter in the bottom material by the bacteria.

A study was now made of the adsorption of bacteria from mixed and pure cultures by the use of relatively large amounts of bottom material. In this experiment, the mud and sand were placed in large test tubes and sterilized by heat at 120° C. for 1 hour. Two volumes of cultured water, 2 days old, or two volumes of a diluted 2-day-old culture of No. 11 were added to the sterile tubes. These were now shaken for 1 minute and allowed to settle for 10 minutes. The supernatant liquid was plated out immediately. The tubes were then allowed to remain in the laboratory for 24 hours; some were shaken

occasionally and some were left undisturbed. The water was plated out after 24 hours. The results (Table III) show that there was again a rapid adsorption of the bacteria from the pure culture by the mud but not by the sand. In the mixed culture there was at first a certain amount of adsorption of the bacteria; this was followed by a very rapid increase in numbers. The large amount of mud used offered a good source of energy, especially after heating, for many of the bacteria in the water.

A comparative study was now made of the adsorption of bacteria by fresh marine mud obtained from Buzzards Bay. Varying amounts

TABLE III

Influence of shaking upon the adsorption of mixed and pure cultures of bacteria by sterile marine bottom material

Nature of culture	Bacteria in 1 cc. of surface water, thousands		
	Shaken		Unshaken
	Start	*24 hours*	*24 hours*
Cultured water control	4.6	153.0	170.0
Plus mud 1329	0.3	3,600.0	39,000.0
Plus mud 1331	0.1	12,000.0	22,000.0
Plus sand	100.0	10,000.0	7,000.0
Bacterium No. 11 * control	33,000.0	214,000.0	130,000.0
Plus mud 1329	3,300.0	4,400.0	31,000.0
Plus mud 1331	30.0	1.0	1.0
Plus sand	39,000.0	320,000.0	220,000.0
Bacterium No. 11 † control	13,000.0	21,000.0	21,000.0
Plus mud 1329	0.7	1.0	1.0
Plus mud 1331	30.0	1.0	1.0
Plus sand	18,000.0	18,000.0	25,000.0

* Dilution of culture with sterile sea water in ratio of 1 : 10.
† Dilution of culture in ratio of 1 : 30.

of the fresh mud, containing 75 per cent moisture, were placed in 250-cc. Erlenmeyer flasks. Some flasks received 100 cc. portions of 2-day-old sea water, while other flasks received 100 cc. of a diluted 2-day-old culture of No. 11. All flasks were shaken for 1 minute, allowed to settle for 10 minutes and the supernatant liquid plated. This was repeated after 1, 2, 4 and 10 days. Some of the flasks were kept undisturbed, while others were shaken at various intervals. The plates were incubated at room temperature for 48 hours and counted.

The results (Table IV) show that fresh marine mud exerted at first a reducing effect upon the numbers of bacteria in the mixed population.

After 24 hours, however, there were more bacteria in the water above the mud than in the control water sample. It is interesting to note that the larger the amount of mud used, the higher was the number of bacteria, especially in the shaken mud, in spite of the fact that the immediate reduction in numbers was greater with an increase in the amount of mud. This was due to the fact that the bacteria found in the mud a source of nutrients and began to multiply rapidly. This confirms the results obtained previously by the use of dry mud and sand.

In the case of the pure culture, there was active adsorption of the

TABLE IV

Adsorption of bacteria by fresh marine mud

Nature of culture	Fresh mud *	Shaking	Numbers of bacteria in 1 cc. water, thousands				
	grams per 100 cc.		*Start*	*24 hours*	*48 hours*	*4 days*	*10 days*
Cultured water .	0	0	53	10	9	9	<1
Cultured water .	2	0	44	17	4	3	<1
Cultured water .	10	0	42	10	32	23	6
Cultured water .	50	0	24	32	50	13	2
Cultured water .	0	+	45	16	11	21	<1
Cultured water .	2	+	30	13	27	29	17
Cultured water .	10	+	33	16	23	36	62
Cultured water .	50	+	23	13	21	79	80
No. 11	0	0	12,700	140,000	240,000	236,000	310,000
No. 11	2	0	11,900	23,000	3,000	2,500	58
No. 11	10	0	13,900	1,000	2,000	3,600	182
No. 11	50	0	17,900	5,900	6,000	1,800	41
No. 11	0	+	14,700	160,000	332,000	310,000	360,000
No. 11	2	+	16,600	67,000	23,000	6,700	160
No. 11	10	+	15,400	13,000	6,000	1,100	92
No. 11	50	+	15,000	4,400	1,000	68	104

* Moisture content of mud 75 per cent.

bacteria by the fresh marine mud, but here also the bacteria began to multiply after 24–48 hours. These were not, however, the typical colonies of the agar liquefying organism added to the mud, but various colonies of bacteria commonly found in the marine bottom.

On the basis of these results, one can hardly conclude that the adsorptive action of the mud bottom upon bacteria in the water is detrimental in any manner to their activities. Even though mud adsorbs, removes, or inactivates a large number of bacterial cells such as may be obtained by growing a pure culture of a bacterium upon an artificial medium, there is still no proof that mud as such is in any manner injurious to bacterial processes in general, either those taking

place in the water or in the mud itself. The evidence presented here seems to point merely to the fact that bottom mud exerts a certain controlling effect upon the nature of the bacterial population in the sea water. This is, of course, true of the bacteria found in the benthos or in the water immediately above it, but may not hold at all for the plankton bacteria found at great distances from the bottom.

The results of these experiments further emphasize the fact that the phenomena of adsorption obtained by the use of pure cultures of bacteria grown upon artificial media are difficult to interpret in terms of natural marine processes, since the conditions controlling the relations of the mud bottom to the natural mixed population of bacteria in sea water are much more complex.

Summary

1. Marine mud exerted an adsorptive effect upon the bacteria in sea water. This effect was particularly evident when certain pure cultures of bacteria were added to the mud to give a heavy inoculation. Sand bottom had very little adsorptive action upon either mixed or pure cultures of bacteria.

2. In the case of the mixed bacterial population in the water, the adsorption of the bacteria by the mud was soon followed by a rapid increase of bacterial numbers; this took place at the expense of the organic matter in the bottom material.

3. These laboratory experiments showed that certain types of marine bottom material exerted a controlling effect on the numbers of bacteria, which were in intimate contact with them. There was no indication, however, of a permanent paralysis of bacterial growth or metabolism.

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PRIMARY SEXUAL PHASES IN THE OVIPAROUS OYSTER (OSTREA VIRGINICA)

W. R. COE

(*From the Osborn Zoölogical Laboratory, Yale University*)

In a recent paper on environment and sex in this species (Coe, 1936*a*), data were presented to show that the sex ratio at the first breeding season is correlated with age and size at sexual maturity. The proportion of individuals first functioning as females was found to be greatest in those localities and during those seasons most favorable for rapid growth. The mean size of the young females is larger than that of males of the same age, as Needler (1932*a*) had previously reported.

During the past year additional evidence has been obtained from other localities along the coast from Long Island Sound to the Gulf of Mexico. This evidence seems to justify certain more or less definite conclusions in regard to the factors which regulate the sexual changes which individuals of this species are known to undergo during their lifetime. Through the courtesy of Dr. Victor L. Loosanoff the writer has had the opportunity of examining additional extensive collections from Milford, Connecticut, while Professor T. C. Nelson has kindly furnished an abundant supply of young oysters of definitely known age from the culture frames of the New Jersey laboratory, on the shore of Delaware Bay, near Cape May. Dr. H. F. Prytherch, of the Bureau of Fisheries, has supplied additional material from Beaufort, North Carolina, and Dr. A. E. Hopkins has sent additional samples from the Gulf Coast laboratory at Apalachicola, Florida. The data concerning the sexual conditions found in this new material are included for comparison with those from other localities in Table I.

Examination of large numbers of fully adult oysters shows that there is usually an approximately equal number of individuals functioning at the time as males and females, or with an excess of females among the older members. The young oysters, on the other hand, always show a large excess of males at their first spawning season. This initial tendency toward maleness is the result of the more rapid growth and multiplication of the male than of the female constituents in the primary reproductive gland in a majority of the young individuals of this alternosexual species (Coe, 1932*a*, 1934).

From Cape Cod to the Chesapeake Bay, most of the young oysters

may be expected to spawn at the age of one year. North of Cape Cod the first spawning is stated to occur more often at the end of the second year, as is the case with some individuals in certain years south of Cape Cod. On the coast of North Carolina and in the Gulf of Mexico, well nourished individuals of the early set spawn toward the end of their first summer, when only three to four months of age, while those of the later set do not become mature until the following spring.

TABLE I

Observed sex ratios during first breeding season at different localities and in different years

Locality	Date	No. undetermined	No. M.	No. H.	No. F.	Total	Ratio F. to 100 M.
New Haven Harbor	7-11-32	17*	389	4	13	423	3.34
" " "	7-27-33	1*	129	0	7	137	5.42
" " "	8-2-35	105*	521	4	40	670	7.66
" " "	7-28-36	3*	327	2	41	373	12.54
Milford (Bed)	8-1-34	2*	80	1	6	89	7.50
" "	7-20-35	2*	312	2	75	391	24.04
" "	7-3-36	77*	780	10	44	911	5.64
" "	8-13-36	7	410	4	58	479	14.15
" "	8-25-36	176†	92	0	21	289	22.83
Milford (Gulf)	7-7-37	0	116	3	25	144	21.55
Milford (Harbor)	7-7-37	10*	306	7	57	380	18.60
" "	7-28-37	6†	116	2	29	153	25.00
Great South Bay, L. I.	7-31-32	21*	197	0	7	225	3.55
West Sayville (float)	1932	0	154	4	48	206	31.17
Delaware Bay	7-7-37	0	520	13	218	751	41.92
Beaufort, N. C.	1933	0	43	0	21	64	48.84
" "	8-10 to 10-20-36	10	84	2	34	130	40.48
" "	10-20-37	73*	151	3	56	283	37.09
Apalachicola, Fla.	1936, 1937	81*	127	0	9	217	7.09
	Total	591	4854	61	809	6315	16.66

* Sexually immature.
† Spawned out.

The ratio of the two sexes at their first breeding season varies greatly from year to year and at different localities, as shown in Table I. It also varies at different parts of the season, not only because the males tend to become mature somewhat earlier than the females but also in consequence of an actual change in the sexuality of some of the individuals because a small percentage of the young oysters function as males in the early part of the season and as females immediately thereafter (Coe, 1936*a*). A few others are hermaphroditic and capable of self-fertilization.

This is well illustrated by three collections of yearling oysters from Milford, Connecticut, kindly supplied by Dr. Victor L. Loosanoff, of the Bureau of Fisheries, in 1936. A sample of 911 yearlings examined at the beginning of the breeding season contained less than 6 recognizable females for each hundred males, in addition to 10 hermaphrodites and 77 small individuals that were sexually immature. At the middle of the season, however, the ratio of females had increased to 14 and near the end of the season to nearly 23 (Table II). In 1937 there was likewise an increase in the proportion of females in the later of two collections from the same locality (Table II). Since the females are often somewhat later in maturing than are the males, they frequently spawn later. Hence, if it may be assumed that the immature individuals found early in the season contained a large proportion of potential females and that the majority of those spawned out when the

TABLE II

Change of sex ratio during first breeding season

Locality	Date	No. undetermined	No. M.	No. H.	No. F.	Total	Ratio F. to 100 M.
Milford (Bed)	7-3-36	77*	780	10	44	911	5.64
" "	8-13-36	7*	410	4	58	479	14.15
" "	8-25-36	176†	92	0	21	289	22.83
Milford (Harbor)	7-7-37	10*	306	7	57	380	18.60
" "	7-28-37	6†	116	2	29	153	25.00

* Sexually immature.
† Spawned out.

last examination was made had previously functioned as males, the differences in the observed sex ratios of the several collections would have an adequate explanation.

An examination of some 4,500 young oysters from Long Island Sound during six successive years has shown a ratio of from $3\frac{1}{3}$ to 25 females for each hundred males, depending upon the local conditions and the season (Table I). The average size of the females exceeds that of the males and as a general rule those seasons and situations most favorable for rapid growth have shown the largest percentage of females.

Different culture beds in near-by situations may differ considerably in the ratio of the sexes in the yearling population. Those in shallow, warmer situations may spawn some weeks in advance of those less favorably situated. The first set may have a more favorable as well as a longer growing season than later ones, with a correspondingly

larger proportion of females. Morphological sex differentiation may occur in the autumn in the one case or it may be delayed until winter in the later sets.

Since there is usually found an approximate equality in the ratio of the sexes or an excess of females after the second year, it is obvious that at least 25 to 45 per cent of the young males in these localities must change to females. It is not yet definitely known whether any of the yearling females change to males but there is conclusive experimental evidence that both males and females sometimes change their sex in the interval between two breeding seasons even at the age of four or more years (Needler, 1932*a*, Galtsoff, 1937). The stimuli which inaugurate these sexual transformations have not been determined, but it is suspected that both inherent tendencies and nutritional conditions may be involved.

The cellular mechanism involved in this change of sexual phase has been shown (Coe, 1932*a*, 1934) to depend upon the presence of gonia of both sexual characteristics or undifferentiated gonia through one sexual phase and their retention as residual cells after spawning. In some species of oysters and other mollusks an alternating rhythm of male and female phases is of regular occurrence (Coe, 1936*c*).

Summarizing the total collections from each area, as shown in Table I, it may be noted that the collections from New Haven Harbor for the four recent years in which suitable material could be obtained comprised 1,066 males and 101 females, a ratio of 9.6 females for each 100 males. From Milford, only nine miles distant, a total of 2,212 males and 315 females were contained in the eight collections during four summers. The sex ratio would there average 14.2 females for each 100 males, indicating a generally more favorable condition for the young oysters than in New Haven Harbor. This conclusion is supported by the fact that the average size of individuals of the same age was somewhat larger at Milford than at the latter locality.

From a natural bed in Great South Bay, Long Island, an exceptionally low ratio of females was found in 1932, while from culture floats not far distant, the ratio was nearly nine times as great (Table I) corresponding with a similar increase in the rate of growth.

Farther to the southward the ratio of females is still greater under favorable conditions, averaging more than forty for each one hundred males both on the culture frames in Delaware Bay and at Beaufort, North Carolina (Table I).

Correlation between Sexuality and Size at First Breeding Season

The more recent studies are quite in harmony with the evidence presented by Needler (1932*a*), Burkenroad (1931*a*), Coe (1932*c*), Roughley (1933) and others that the mean size of individuals functioning as females at the first breeding season is considerably greater than that of males of the same age. In an unselected sample consist-

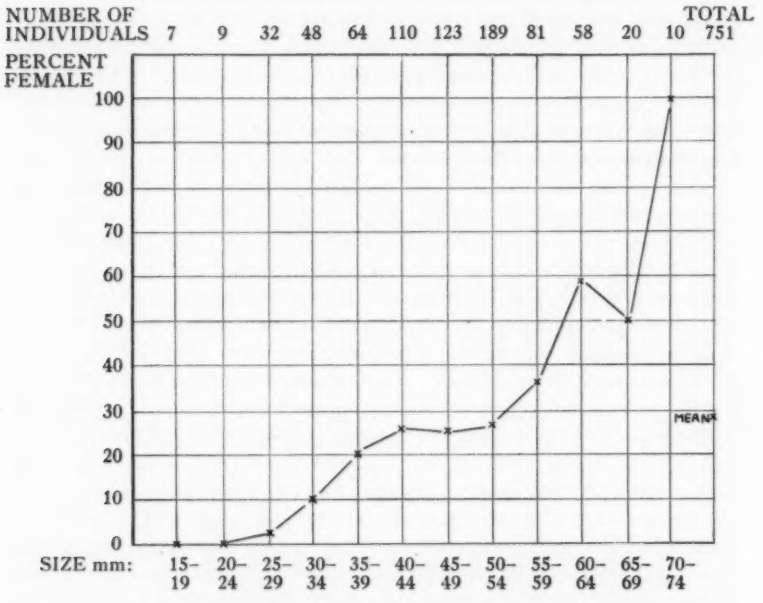

Fig. 1. Graph showing correlation between sexuality and size at first breeding season, based on an unselected sample of 751 yearlings from the culture frames at the New Jersey laboratory on the Shore of Delaware Bay near Cape May. The size of the class is shown at the bottom of the graph and the number of individuals in each class at the top. The mean percentage of females in the entire sample is also indicated.

ing of 751 yearlings from the culture frames at the laboratory on the shore of Delaware Bay, 358 had a shell length of 50 mm. or more on July 7, 1937, while 393 were smaller. Examination of the gonads showed a ratio of 63.4 females for each 100 males in the group of larger individuals as compared with 26.6 females for each 100 males among the smaller ones. This correlation between sexuality and size for these 751 young oysters is shown graphically in Fig. 1. It will be noted that none of the females was less than 25 mm. in length and

none of the males more than 69 mm. The largest number of individuals, however, were between 40 and 55 mm. in length and in these groups there was little difference in the percentage of females.

Direct Female Development

The primary gonad may be either distinctly bisexual in appearance or it may become differentiated very early into either of the two sexual phases (Coe, 1932*a*). In the latter case there is a direct transformation of the primary undifferentiated gonia into the functional sexual cells. The female phase is then attained without indication of protandry.

In Long Island Sound the sexual phase of the first breeding season is sometimes distinguishable as early as October or November in exceptionally well nourished young at the age of only three or four months after setting. More frequently, however, morphological sex differentiation is delayed until mid-winter or later.

At Beaufort, North Carolina, direct female development appears to be the general rule, with a much higher ratio of young females than in more northern localities (Table I). Samples of well nourished individuals from Delaware Bay, known to have set July 9, 1936, likewise indicated direct development, when examined at the end of December. No evidence of incipient protandry was found at that time, although in many cases the gonads were sexually differentiated.

Indirect Female Development

It has been shown previously (Coe, 1932*a*), that the primary gonads of some young individuals develop directly into ovaries or spermaries, as the case may be, while those of other individuals of the same age are distinctly bisexual, or, to use a less ambiguous term, ambisexual.

The latter are characterized by a cortical layer of ovogonia and young ovocytes, with spermatogenic cells at one side or in the lumen. In many cases these ovocytes later degenerate or they may remain inactive during the primary functional male phase, to become activated and functional toward the end of that phase or some time thereafter. In this way the sexual phase may change from male to female either during the first breeding season or, much more frequently, during the following autumn.

A subsequent change from female to male phase evidently results from the later activation of some of the descendents of the primary undifferentiated gonia into spermatogonia. The propagation of such undifferentiated gonia may be continued year after year or even through a long lifetime, since some residual gonia always remain after spawning.

It was also previously reported (Coe, 1932*a*) that the developing gonads exhibit all intergradations between those of the so-called true males in which no indications of ovogonia or ovocytes can be detected and those which develop directly into ovaries. In a few individuals both types of cells in the ambisexual gonad multiply harmoniously, leading to the functionally hermaphroditic condition at the breeding season.

In northern localities some of the spermatogonia in the young ambisexual type of gonad may undergo transformations during the autumn comparable with those taking place in normal spermatogenesis (Coe, 1932*a*). These cells may later degenerate, followed by the activation of ovogonia, thus leading to the functional female phase at the first breeding season during the following summer. Such incipient protandry seems to be much less frequent than the direct development in which the initial dominant phase, either male or female, is retained during the winter and the following breeding season.

Sexual Conditions in Southern Localities

From North Carolina southward the initial sexual phase in the earliest set of the year becomes functional toward the end of the same season, when the young oyster is only three or four months of age, as Burkenroad (1931*a*) reported for the Louisiana oyster. Young of the later sets are similar to those of more northern localities in their sexual conditions, although protandry may be less frequent (Table I). Thus at Beaufort, North Carolina, and at Apalachicola, Florida, on the Gulf of Mexico, there may be two generations of this species in one year, individuals of the early set spawning some three to four months after setting while those of later broods do not become ripe until the following spring.

Cultures in both these localities are usually contaminated with more or less numerous individuals of the larviparous species, *O. equestris*, which matures at a still younger age and has a breeding season covering most of the year in warm situations. It is difficult to distinguish the two species externally when young, but the relations of the epibranchial chamber differ and the gonads are very different, since *O. equestris* has a sequence of overlapping male and female sexual phases and the spermatogenic cells are larger than in *O. virginica* and arranged in dense clusters.

A small collection of the over-wintered young at Beaufort in 1933, kindly supplied by Dr. H. F. Prytherch, showed a ratio of nearly 49 females to 100 males, while the first set of the season in 1936, stated to have occurred about June 1, showed a female ratio of about 40 when

examined in August, September and October. The largest individuals had a shell length of 23 mm. in August, 40 mm. in September, and 70 mm. in October. In August there were only a few males with ripe sperm and toward the end of October most individuals of the early set were spawned out.

Collections of the early set taken September 20, 1937, about fifteen weeks after setting, were from 20 to 50 mm. in length and showed a female ratio of 37.09 (Table I). Those which exceeded 35 mm. in length had a female ratio of about 61 as compared with 16 for those of smaller size.

An unselected sample containing 88 individuals which were one year old, originating from the autumnal set during the last two weeks in September, 1936, on the other hand, showed a ratio of 75 females to 100 functional males. These were doubtless in their second spawning season, having previously participated in the spring spawning during June, 1937, when seven to eight months of age. The ratio of females was thus about twice as great as was found in the young at the first spawning period. This would indicate that the proportion of young males changing to the female phase for their second spawning period must have been about 22 per cent greater than the number of individuals, if any, which experienced a sex change in the opposite direction during the same time.

Small collections from Apalachicola, Florida, obtained through the courtesy of Mr. R. O. Smith, were examined three to five months after setting in 1936. These, combined with an additional collection kindly supplied by Dr. A. E. Hopkins from the same locality in 1937, showed a female ratio of 7.09 (Table I), corresponding closely to the ratios from northern localities under unfavorable conditions.

Discussion

The foregoing evidence concerning the sexual conditions in the Virginia oyster indicates that the sex-differentiating mechanism must be in a very labile condition since it responds so generally to environmental influences.

Because of the different sex ratios under different environmental conditions, it seems probable that the responses noted may depend upon the interaction of several associated factors, both genotypic and phenotypic. Among these it is evident that all these populations have at least the three following categories of hereditary and environmental influences.

(1). There is an inherent tendency toward protandry, as is the case with so many other mollusks, both pelecypods and gastropods,

including the larviparous oysters (Orton, 1927; Coe, 1932*b*), *Teredo* (Coe, 1936*d*), *Venus* (Loosanoff, 1937), *Patella* (Orton, 1928), and *Crepidula* (Coe, 1936*b*, 1938), to mention only a few of the numerous examples. This tendency may or may not be realized, since direct female development so frequently occurs under favorable conditions.

(2). There appears to be an inherent tendency toward rhythmical change in the sexual phase during successive breeding periods, as in the larviparous oysters, *Teredo*, and certain gastropods. The fully adult Japanese oyster (*Ostrea gigas*) was proved by Amemiya (1929) to be subject to this change in sexuality in the interval between two breeding seasons. In the Virginia oyster a corresponding condition has been found by Needler (1932*a*, 1932*b*), by Burkenroad (1937) and by Galtsoff (1937). The proportion of the adult population in which this change occurred varied greatly in the different experiments, the discrepancies being due presumably both to varying environmental conditions and to comparatively small samples. The most extensive of these experiments is that by Galtsoff, who found that among 202 fully adult oysters of which the functional sexuality was reliably determined during the breeding season of 1936, only 167 retained the same sexual phase in 1937. Of the remainder, 14 had died in the meantime, while 9 or 14 per cent of the females had become functional males, and 11, or 8.9 per cent of the males were found to be in the female phase in 1937. The twenty individuals which experienced this change of sexuality during the interval between the two breeding seasons represented only 10.7 per cent of the total number of survivors.

It is evident, then, that sexual stability may predominate over any tendency toward rhythmical change much more definitely than is the case with the larviparous species. There is also considerable evidence which indicates that the sexual rhythm in the latter is associated with a metabolic rhythm (Coe, 1934). But the nutritive conditions are certainly modified by environmental influences. Change of sexual phase in *Crepidula*, like metamorphosis in other animals, has been shown recently (Coe, 1938) to be subject to environmental control, and this is presumably true to some extent in the oyster.

Immediately after spawning, the residual gonia in both the sexual phases show considerable variation. Some individuals which have spawned as males show residual small ovocytes in addition to apparently undifferentiated gonia. Others show only spermatogonia and undifferentiated gonia, while females may retain few or many or no young ovocytes after the cytolysis of the larger ovocytes. In both sexual phases cytolysis may proceed until only undifferentiated gonia remain and the individual has become essentially neutral.

Evidently relatively few fully adult individuals have gonads that are so evenly balanced that slight changes in the internal environment may activate either type of the residual gonia and thus determine the sexual phase of the following season. In the majority of both sexes, the same sexual phase is retained (Amemiya, 1929; Needler, 1932*a*; Galtsoff, 1937; Burkenroad, 1937).

(3). Since the primary gonads in the young oyster before morphological sex differentiation show great variability in the proportion of cells characteristic of the two sexual phase, it seems probable that these are to some extent at least dependent upon different heritable modifying factors (Coe, 1932*a*, 1934, 1936*a*). Functional males, hermaphrodites and females may then be expected under uniform conditions, the proportion of each depending upon the combination of factors present in each individual. But in the population of young oysters in any locality preceding the time or primary sexual differentiation, in the autumn, winter or summer, according to the locality, even a generally uniform environment will be highly variable in so far as each individual is concerned, since there are differences in age, in situation relative to other individuals and the like (Orton, 1936).

The larger average size of the females at the time of sexual differentiation and later and the higher ratio of females in those years and in those localities or situations most favorable for rapid growth may indicate either (*a*) differences in hereditary factors or (*b*) a direct response sexually to nutritive and other conditions, or (*c*) both. The evidence to date seems to imply that both these influences are operative.

The sexual conditions in other species of oviparous oysters are similar to those here discussed for the Virginia oyster. In the Indian oyster (*O. cucullata*) Awati and Rai (1931) found a corresponding tendency toward protandry, but with an excess of females in the adult population, which averaged 41 per cent males, 3 per cent hermaphrodites and 56 per cent females. Those which harbored the commensal crab *Pinnotheres*, however, showed 82 per cent males, 7 per cent hermaphrodites and only 10 per cent females, indicating a masculinizing influence on the part of the commensal, either nutritive or otherwise. Roughley (1933) likewise found a very strong tendency toward protandry in the Australian oyster (*O. commercialis*), but the adult population consisted of 54 to 88 per cent females, averaging about 2.7 times as many females as males. He considered that nutritive conditions were not influential in determining the sexual phase, since populations under poor conditions for growth showed about the same ratio of females as those having better nutritive conditions. The Portuguese oyster (*O. angulata*) is another species in which the females

are more numerous than the males in the adult population, Orton and Awati (1926) reporting ratios of 60 to 88 per cent females. Indeed, in relatively few species of fully adult pelecypods are the males as numerous as the females (Pelseneer, 1935).

Amemiya's studies on change of sex in the Japanese oyster (1929) include later experiments (1935) in which a portion of the gills was removed from adult oysters immediately after spawning. The operation was performed on four groups of oysters, with an equal number of controls. The following year there was a slightly larger ratio of males in each one of the operated groups than in the controls. It was concluded that the proportion of individuals assuming the male sexual phase was influenced by the decreased facility of the operated individuals in obtaining food.

All these considerations emphasize the complexity of genotypic and phenotypic factors which may be influential in controlling the sexual phases of the Virginia oyster. The evidence here presented indicates that the populations along the coast from New England to the Gulf of Mexico consist of several local races living under widely different environmental conditions and differing in some respects in their inheritance. In the more northern localities a certain proportion of the young oysters function as females during their first breeding season at the age of about twelve months either by direct female development or indirectly after passing through an abortive or, less frequently, a brief functional male phase. In southern localities two generations, of which the second comprises a large percentage of females, may occur within the same twelve months period.

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THE DELAYED OCCURRENCE AND TOTAL OMISSION OF ENDOMIXIS IN SELECTED LINES OF PARAMECIUM AURELIA

T. M. SONNEBORN

(From the Department of Zoölogy, Johns Hopkins University)

Experimental control of the process of endomixis in *Paramecium aurelia* has been only partly achieved. Jollos (1916), Sonneborn (1937*b*) and others have developed methods of inducing endomixis, but no method of avoiding it completely is known. Temporary suppression of endomixis was probably attained by Jollos (1916); but the method employed volumes of culture medium so great as to make it impossible to ascertain with reliability whether endomixis was occurring or not. The present paper sets forth a method that avoids this difficulty and provides not only some lines of descent with greatly extended interendomictic intervals, but also others that never go into endomixis.

The method is based on the observation (Sonneborn, 1937*a*) that the interendomictic interval varies greatly in sister lines cultivated under the same conditions. An attempt was therefore made to select the lines with the longer intervals. This was done by discarding lines as they went into endomixis and replacing them by new lines begun with surplus individuals from sister lines that had not yet gone into endomixis. When this is done with a group of 12 to 24 daily isolation lines cultivated under the conditions employed by Sonneborn (1936), the following phenomena occur. At first, none of the lines go into endomixis; then, as the "normal" time for endomixis approaches, more and more of the lines go into endomixis and have to be replaced by their sister lines that have not yet gone into endomixis. During a period of several weeks, this high frequency of endomixis continues and necessitates equally frequent eliminations and replacements; but during the following few weeks the frequency of endomixis greatly decreases and thereafter occurs but rarely. Selection has thus been effective in seeking out lines in which endomixis completely fails to occur. However, such lines cannot be continued indefinitely; after four or five months they die.

This method has been successfully applied in this laboratory many times and is now a routine technique. As illustrations of the results obtainable with it, two typical histories will be given in the following

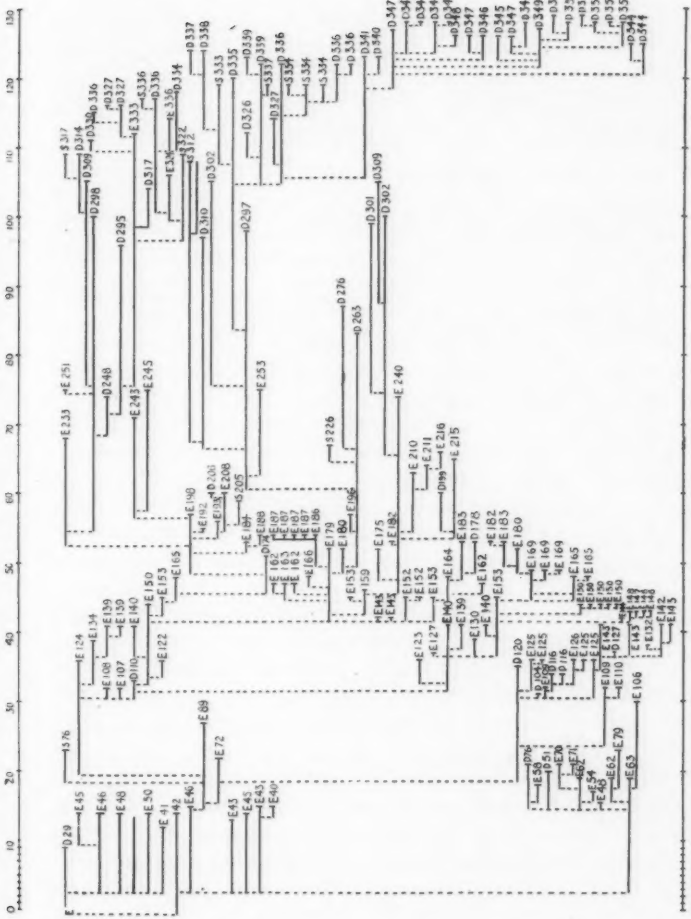

FIG. 1. Selection of non-endomictic lines in race *R*, *Paramecium aurelia*. The solid horizontal lines represent daily isolation lines of descent. The broken vertical lines connect sub-cultures with their source cultures. *E* symbolizes endomixis; *D*, died; and *S*, killed and stained after two days without fission. The numbers following *E*, *D*, and *S* give the number of fissions without endomixis in the direct line of descent from the original progenitor in endomixis on the first day to the final individual which terminated each sub-culture.

two sections: one of these involves animals of the Johns Hopkins race *R*, reported upon in recent papers by the author and associates; the other involves animals of Woodruff's long-lived race, here designated *W*. In the third section, the relation of these results to the variability of the interendomictic interval is set forth.

Race R

Figure 1 gives the results of applying the method of selection to a group of typical lines of race *R*. The entire group was derived from one individual in endomixis April 14, 1935. The original 12 lines descended from this individual went into endomixis between the 14th and 20th days (after 41 to 63 fissions) following the initial endomixis.

TABLE I

The frequency of endomixis and of death in relation to the time since the last preceding endomixis in races R and W of *Paramecium aurelia*

Race	Days since preceding endomixis	Number of lines in endomixis per 100 line-days	Number of deaths per 100 line-days
R	1–13	0	0.8
	14–55	23.3	2.6
	56–76	6.4	2.4
	77–122	0.6	5.9
	123–130	0	35.8
W	1–30	0	2.0
	31–76	3.8	0.8
	77–130	0.5	4.8
	131–163	0	13.4

Each of these lines was replaced at the time of endomixis by a new line started with a surplus individual from one of the sister lines that had not yet gone into endomixis. By repeating this process of replacement in all the descendant lines whenever endomixis occurred or might have occurred, some of the lines lived for as much as 130 days (350 fissions) without endomixis.

As shown in Table I, the life of this group of lines fell into five periods differing markedly in the frequency of endomixis and death. The first period, extending to the 13th day, was characterized by the complete absence of endomixis. In the second period, from the 14th to 55th days, many lines went into endomixis and had to be replaced. The third period, from the 56th to 76th day, was marked by a great reduction of the number of lines going into endomixis. In the fourth period, from the 77th to 122nd day, only three lines went into endo-

mixis, but the number that died or stopped multiplying increased. Finally, in the fifth period, from the 123rd to 130th day, no line went into endomixis, but the death rate was so high that the group completely died out. In Table I, lines which stopped multiplying and were found, on staining, not to be in endomixis, are included among those which died. Experience showed that this was their usual fate.

The main feature of the preceding account is that endomixis did not occur in certain lines of descent carried through 350 fissions during 130 days. This was demonstrated by cytological studies. Each line of descent was stained on every day that fission occurred; and if fission failed to occur in any line for two successive days the line was stained and so brought to an end. Since every line that showed any nuclear condition even remotely suggesting endomixis was discarded, it is certain that no endomixis was overlooked and that the lines living through till the end of the experiment had not experienced endomixis during the entire time.

Race *W*

The method of obtaining non-endomictic lines of descent was also applied successfully to race *W*, though here the detailed results differ from those obtained with race *R*. A typical set of results on race *W* is illustrated by Fig. 2. The group there represented began with four individuals in endomixis on February 10, 1935. As shown in Table I, the life of this group is divisible into four distinct periods. In the first period, extending to the 30th day, there was no endomixis and few deaths occurred; in the second period, from the 31st to the 76th day, the frequency of endomixis was at its highest and the death rate was still low; in the third period, from the 77th to 130th day, there were few endomixes (two certainly, and possibly four), but the death rate showed a marked increase; in the fourth period, from the 131st to 163rd day, there were no further endomixes and the death rate reached its peak, resulting in the extermination of the group.

As in race *R*, the lines of race *W* which were carried to the end of the experiment showed no endomixis during the entire period of observation. Their life without endomixis extended for 163 days and included 302 successive fissions. This long period is $5\frac{1}{2}$ times as long as those reported to be typical for this race by Woodruff and Erdmann (1914) and it includes about seven times as many fissions.

Comparison of the data for the two races given in Table I shows that the group of race *W* lines had a longer initial period without endomixis (30 days as compared with 13), and that it at no time attained so high a rate of endomixis or death as did the group of race *R* lines. Further, the maximum period without endomixis was longer in race *W* than in race *R*, but included fewer fissions.

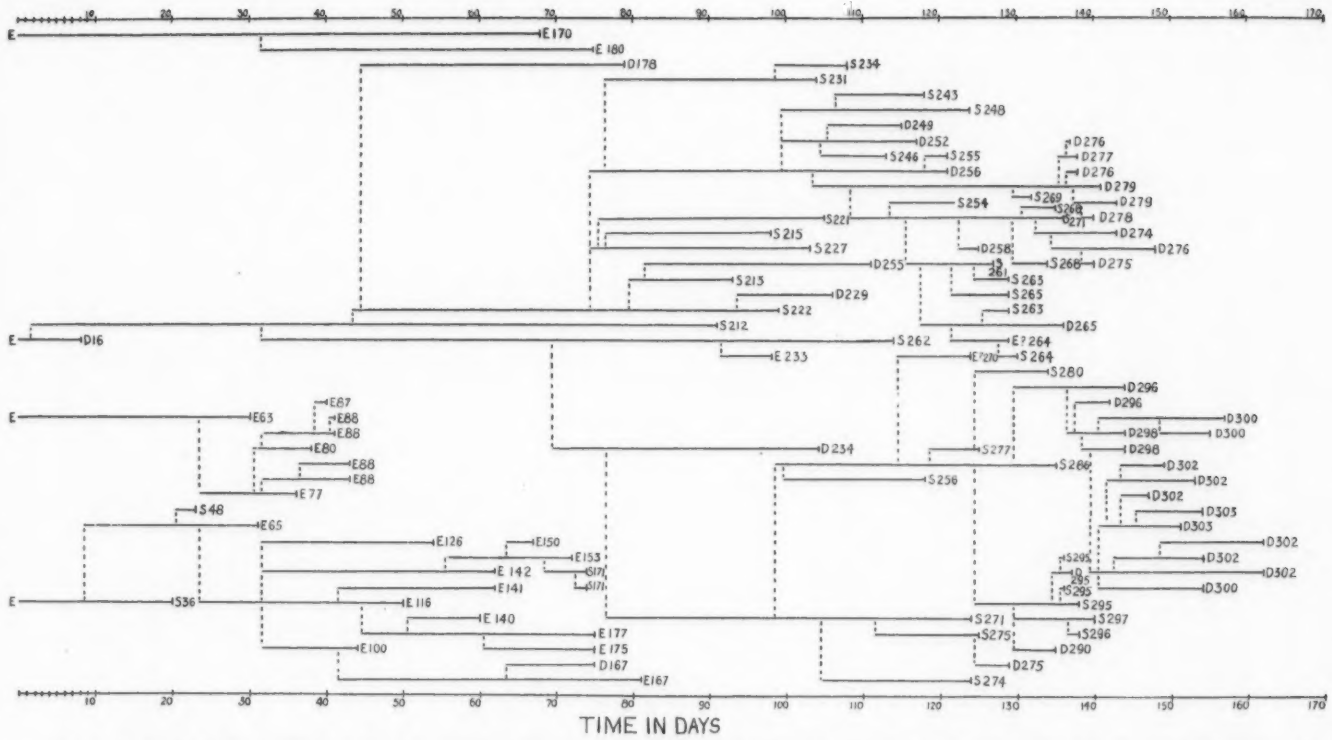

FIG. 2. Selection of non-endomictic lines in race *W*, *Paramecium aurelia*. The solid horizontal lines represent daily isolation lines of descent. The broken vertical lines connect sub-cultures with their source cultures. *E* symbolizes endomixis; *D*, died; and *S*, killed and stained after two days without fission. The numbers following *E*, *D*, and *S* give the number of fissions without endomixis in the direct line of descent from the original progenitors in endomixis on the first day to the final individual which terminated each sub-culture.

Three other races (*H*, *E*, and *S*) have been subjected to the same method of selection with similar results. It seems reasonable to suppose that what has been accomplished in these five races could probably be done with most or all races of *P. aurelia* under similar cultural conditions: by selection, lines can be isolated that do without endomixis throughout their lives—a period 5 to 7 times as long as the ordinary interval between successive endomixes.

Variability of the Interendomictic Interval

In the absence of selection, the extent of the period during which no nuclear reorganization occurs was shown by Sonneborn (1937*a*) to vary greatly. The preceding sections show that still greater variability appears when the selection technique is applied. This greater variability must be due to the existence of relatively rare lines which, without any special experimental treatment, have extremely long interendomictic intervals or even fail completely to undergo endomixis. Since such lines are very rare, they would ordinarily not be found in work where the investigator is able to observe only a small sample of a race. The method of selection serves simply to seek out these lines and multiply them. Without resorting to any change of cultural conditions, it provides a means of obtaining both lines that long omit endomixis and lines that omit it entirely.

Summary

If daily isolation lines of *P. aurelia* are regularly discarded as soon as they go into endomixis, and if these are then replaced by sister lines that have not yet gone into endomixis, it is possible to maintain for long periods lines which have not been in endomixis since the start of this procedure. With this method there were obtained lines of race *R* which omitted endomixis for as long as 130 days and 350 fissions, and lines of race *W* which omitted endomixis for 163 days and 303 fissions. At the end of these long periods, all lines died.

In the culture of groups of lines selected in this way, there is an initial period during which endomixis does not occur; this is followed by a period in which endomixis occurs in many of the lines (which have to be discarded); during the remaining history, endomixis occurs rarely and eventually not at all, but the death rate rises and results in the final extinction of the group. The details as to the duration of these periods and their characteristic death and endomixis rates differ in different races. The method of selection emphasizes the enormous variability of the interendomictic interval. The extremely long intervals are very rare, and the selection method is simply a device for finding these rare lines and multiplying them.

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THE DISTRIBUTION OF AMMONIA IN THE WATERS OF THE GULF OF MAINE

ALFRED C. REDFIELD AND ANCEL B. KEYS

(From the Woods Hole Oceanographic Institution[1] and the Biological Laboratories, Harvard University)

In the decomposition of organic residues in the sea ammonia is apparently the first simple inorganic compound of nitrogen to be set free. Since it does not commonly occur in large quantities, it is evidently oxidized rapidly to nitrate and nitrite. As an ephemeral intermediate in the cycle of regeneration of nitrate, it is of interest in indicating the places in which the decomposition of nitrogenous material is taking place.

The present paper presents the results of a survey of the occurrence of ammonia in the waters of the Gulf of Maine based on observations made during cruises of the "Atlantis" in September, 1933 and May and June, 1934. The only previous measurements of ammonia in these waters of which we are aware are those made by Seiwell (1931) in Penobscot and Frenchman's Bays and those recorded by Rakestraw (1936) in a study of the occurrence of nitrite in the sea. Our results are correlated with the studies of nitrite made by Rakestraw of the phosphorus cycle by Redfield, Smith and Ketchum (1937), and with observations on the distribution of plankton, based on collections made during these cruises.

Methods

Ammonia was determined by the Teorell titration of the vacuum distillate as described by Krogh (1934). The apparatus could be used practically on shipboard, using alcohol lamps burning pure alcohol for heating and a Cenco Hyvac pump as a source of low pressure. Ammonia-free water was prepared ashore. It was found necessary to steam out the apparatus before the start of each series of analyses and to make blank analyses between each pair of unknowns. To be reliable, all determinations must be made in duplicate, since unforeseen contamination frequently occurred.

The sea water samples were taken in Nansen reversing bottles, from which they were immediately transferred, with double rinsing, to glass-stoppered bottles. These were stored in the refrigerator and

[1] Contribution No. 159.

analyzed as quickly as possible. Experiments recorded in Table I showed that sea water stored for about eight hours underwent no appreciable change in ammonia content. With longer periods of storage there is a progressive decrease in the ammonia content, which is not prevented by the addition of 0.1 per cent mercuric chloride (Keys, Christensen and Krogh, 1935). The reason for this loss is unknown.

Results

The positions of the stations at which reasonably complete and satisfactory observations on the distribution of ammonia were obtained

Table I

Stability of ammonia in stored sea water samples *

First analysis		Second analysis		Third analysis	
Hours after collection	NH_3-N, mg./M^3	Hours after collection	NH_3-N, mg./M^3	Hours after collection	NH_3-N, mg./M^3
1	40	7	41	290	10
2.5	27	8.5	19	290	8
3.0	35	7	35	290	15
2.5	28	8	30	290	2
4	20	—	—	90	13
4	62	17	54	90	45
6	42	—	—	90	25
6	44	18	24	—	—
6	57	18	46	90	37
5	49	17	28	90	23
4	46	17	32	—	—
5	42	18	30	90	20
†1.5	36	290	8		
†2	25	290	4		
†2.5	21	290	15		
†8.5	31	290	16		

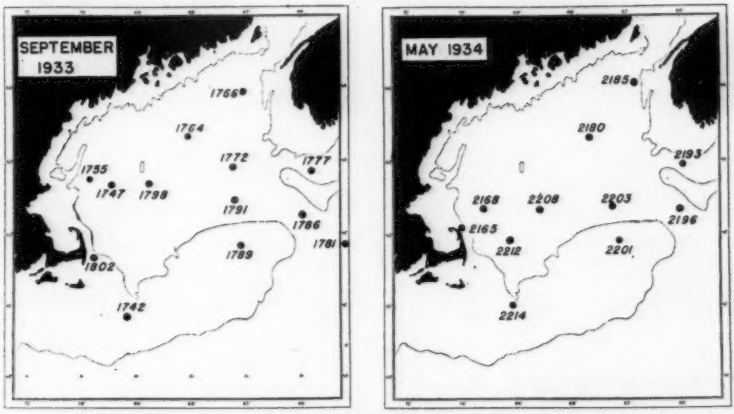
* All values are averages from duplicate analyses.
† $HgCl_2$ added to sample at time of collection.

in September, 1933 and May, 1934 are shown in Figs. 1 and 2. Observations were also made at six stations in the Gulf between June 25 and June 30; the results obtained at that time did not differ from those of a month earlier except that on the whole smaller amounts of ammonia were present. In presenting the results it is convenient to separate the observations made in deep water where the stability of the water column preserves its heterogeneity from those obtained in shallow water and in the shallower channels across the banks where a high degree of turbulence prevails.

Observations in Deep Water

Figure 3 represents the concentrations of ammonia at various deep stations in September and in May. The stations are arranged as far as possible in the order of the positions through which the water is thought to circulate on penetrating the Gulf. Stations occupied in May are placed under stations occupied in comparable positions in September.

In September, Station 1781, 45 miles southeast of the Eastern Channel, showed the presence of some 10 mg. N per cubic meter in the upper 60 meters, diminishing to zero at 100 meters, below which level no ammonia was detected down to the depth of 2,000 meters. The ammonia in the water in the Eastern Channel was similar in distribution, there being none observable below 125 meters. Within the



Figs. 1 and 2. Position of stations at which distribution of ammonia was determined in September, 1933 and May, 1934 respectively.

Gulf, on the other hand, the concentration of ammonia was distributed rather uniformly throughout the water column. The concentrations of ammonia occurring at any station increased towards the western basin of the Gulf, where amounts exceeding 50 mg. N per cubic meter were found.

In May the distribution of ammonia was very different. Amounts in excess of 10 mg. N per cubic meter were rarely observed in the upper 20 meters and at depths greater than 100 meters. Between these depths, at 30 to 60 meters, maximal concentrations occurred ranging in value from 10 mg. N per cubic meter in the Eastern Channel to 45 mg. N per cubic meter in the southern part of the western basin. In the following month similar conditions were observed save that the maximal concentrations did not exceed 25 mg. N per cubic meter.

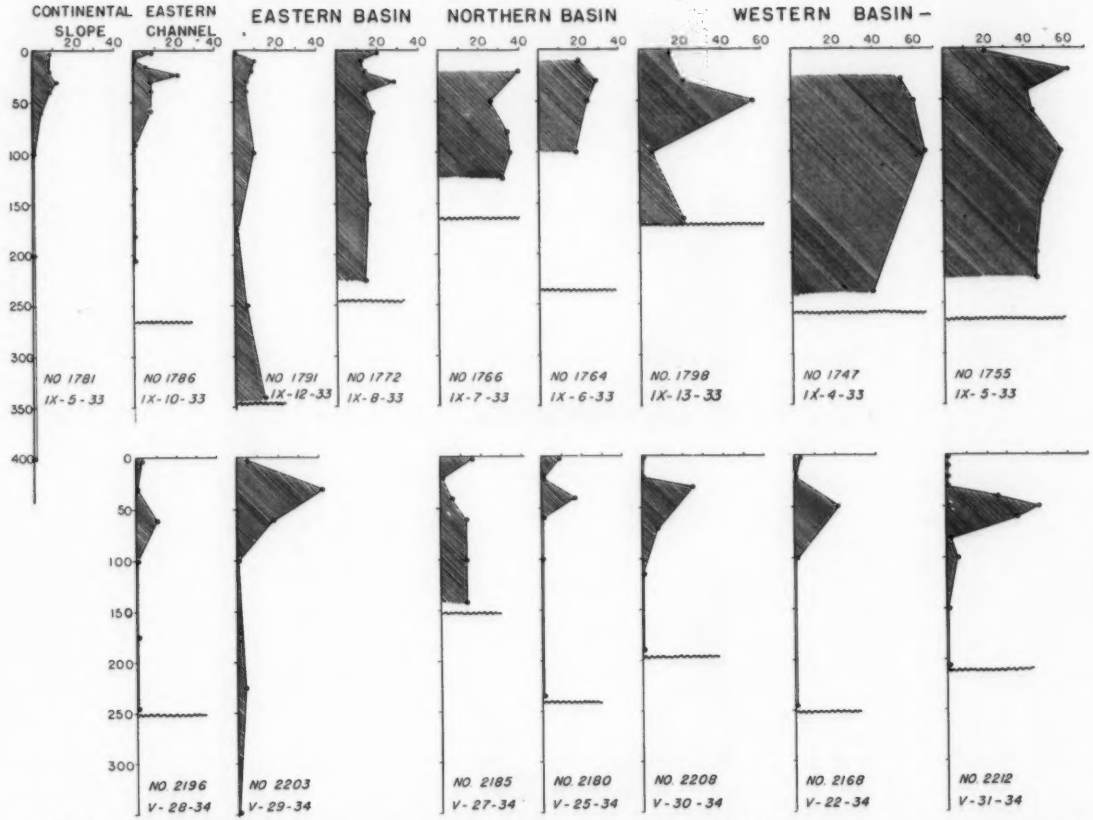


FIG. 3. Vertical distribution of ammonia in the deeper waters of the Gulf of Maine. Upper row, in September, 1933; lower row, comparable positions in May, 1934. Depths measured in meters downward along the ordinate. Concentrations of ammonia expressed as milligrams ammonia nitrogen per cubic meter along the abscissa.

Observations in Shallow Water

Figure 4 shows the distribution of ammonia at shallow stations off Cape Cod and on Georges Bank and in the strong tide ways of the South and North Channels. No definite difference in the seasonal picture is evident. In the channels considerable concentrations of ammonia occurred at all depths, though in May a maximal concentration a short distance below the surface was present in the North

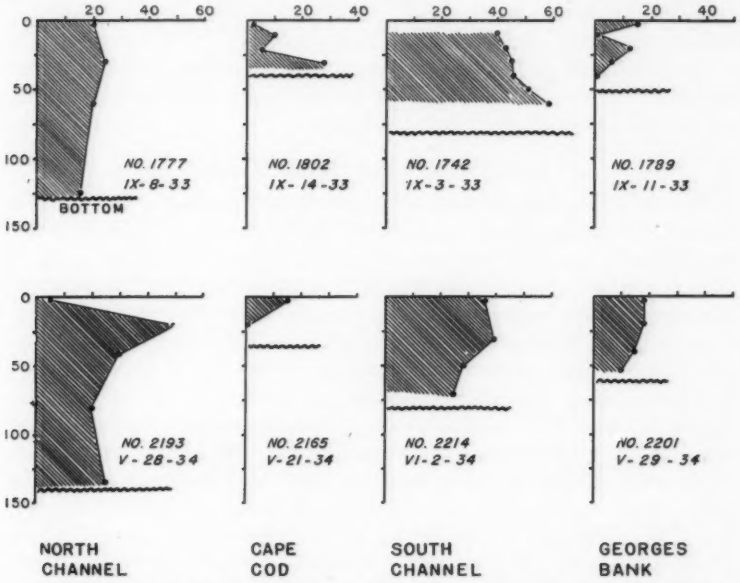


FIG. 4. Vertical distribution of ammonia in shallow waters of the Gulf of Maine. Upper row, in September, 1933; lower row, comparable positions in May, 1934. Concentrations of ammonia expressed as milligrams ammonia nitrogen per cubic meter along the abscissa.

Channel. In the shallow stations the concentration sometimes increased with depth, sometimes the reverse.

Discussion

The distribution of ammonia appeared very irregular when the observations were first made. The magnitude of the concentrations of ammonia observed agreed well with the general range of observations made by earlier workers in various regions (Robinson and Wirth, 1934). It appeared, however, that great differences might be observed

in similar situations at any time and in the same situation at different times.

In several ways our observations support the conclusions drawn from similar studies of ammonia in the English Channel made by Cooper (1933). He considers the formation of ammonia to be a "surface" phenomenon with lesser activity at the bottom. In the spring the surface concentrations decrease from utilization by phytoplankton. Our May observations would appear to be adequately explained on these assumptions. He also observed a general increase in summer and autumn, as did we in September.

As additional evidence has accumulated concerning the biology and chemistry of the Gulf, a number of correlations appeared which seem to give the findings additional significance.

Relation to Phosphate Cycle

The most striking difference between the occurrence of ammonia in September and in May is its relative abundance in the deep water at the end of summer compared with its absence there in the spring. This is precisely the relation found for another intermediate product of decomposition—dissolved, organic phosphorus compounds. These were found by Redfield, Smith and Ketchum (1937) to disappear from the water of the western basin in mid-winter and gradually to accumulate during the summer, reaching a maximum observed concentration in November. Like the ammonia in September, the concentration was quite uniform at all depths. In the spring these phosphorus compounds appear in the subsurface layers, as does the ammonia, before they may be detected in considerable quantity in the depths. It will be of interest to observe whether the ammonia does not almost entirely disappear from the water in mid-winter, as does the dissolved organic phosphorus.

Like the organic phosphorus, ammonia appears to accumulate at all depths in late summer. This should not be thought of as the accumulation of an inert product, however, for von Brand, Rakestraw, and Renn (1937) have shown, at least in a laboratory experiment, that the ammonia of decomposing plankton is nitrified completely in 30 days. It is probable that the ammonia is formed by decomposition throughout the water column, for in September there are no well-marked gradients of ammonia concentration which are necessary for its transport by eddy conductivity. There is no evidence, except in a few of the shallow stations, of increasing concentrations near the bottom, such as Seiwell (1931) observed in shallow bays and as might occur if decomposition on the sea bottom was responsible for a considerable part of the regeneration of nitrogen.

Relation to Nitrite

If the view to which Rakestraw (1936) has recently lent support is correct—that nitrite arises in the water through the oxidation of ammonia—one would expect a close correlation in the distribution of these nitrogen compounds. This expectation is in part fulfilled. The distribution of ammonia in May closely parallels that of nitrite, as shown in Fig. 5. Both may be explained through decomposition taking place in the subsurface waters. Rakestraw explains the absence of nitrite from the water immediately below the surface as being due to

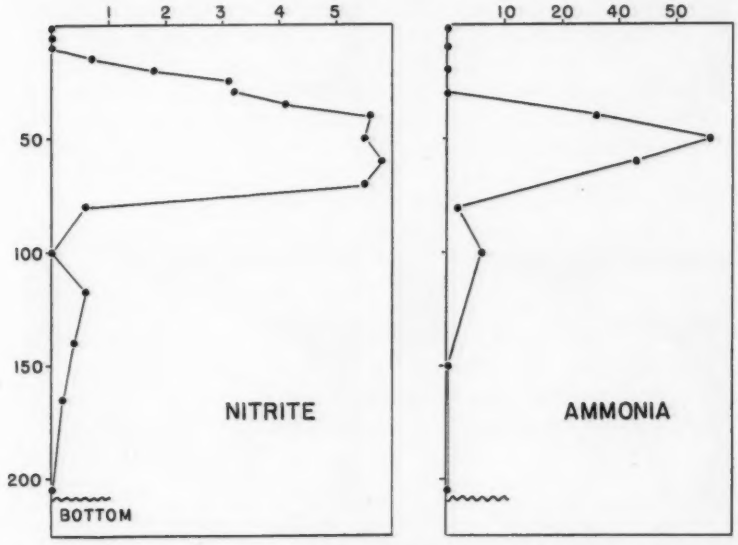


Fig. 5. Vertical distribution of nitrite and ammonia at Station 2212 May 31, 1934. Depths measured in meters downward along the ordinate. Concentrations measured in milligrams nitrogen present as nitrite or as ammonia per cubic meter.

the assimilation of this substance by plankton. Evidently ammonia is similarly consumed. It is interesting, though not surprising, that there is no evidence that soluble organic phosphorus compounds are assimilated in a similar way—since they were found in as high concentration in the surface as at any depth.

Rakestraw remarks that the maximum for nitrite occurs between June and September. However, the nitrite does not appear to accumulate toward the end of summer to the extent to which ammonia and organic phosphorus compounds do. Nitrite is evidently a shorter-

lived link in the chain of nitrogen transformations than is ammonia for even in May the ammonia nitrogen concentration exceeds that of nitrite nitrogen by ten-fold.

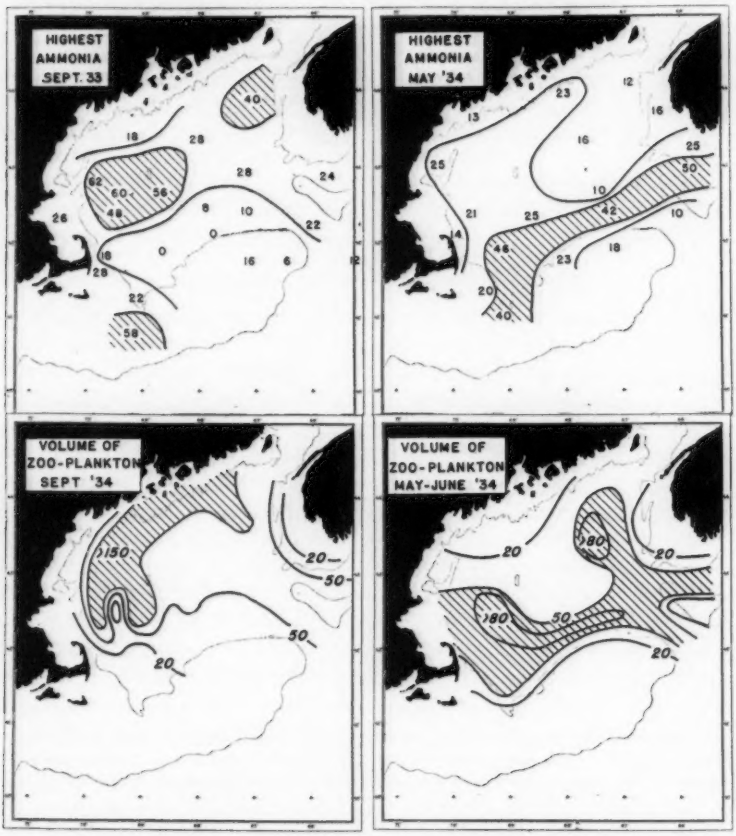


FIG. 6. The highest concentration of ammonia in the upper 80 meters of water in various parts of the Gulf of Maine in September, 1933 and May, 1934 compared with the quantitative distribution of zoöplankton. Ammonia concentrations expressed in milligrams ammonia nitrogen per cubic meter. Zoöplankton volumes expressed as cubic centimeters of dry plankton per square meter of surface area caught in a vertical haul from near the bottom.

RELATION TO PLANKTON

The quantity of ammonia observed during both cruises varied greatly from station to station. In September the greater quantities

were observed in the northern and western parts of the Gulf; in May in the southern and eastern regions. These facts did not assume significance until the plankton catches made at the time had been analyzed.

During each cruise a standard vertical zoöplankton haul was made at each station occupied (a number far exceeding those at which the ammonia was investigated). A 1.5-meter silk net was hauled from near the bottom to the surface. The total catch at each station was freed of excess moisture on a filter and its volume measured by displacement. The results of these measurements expressed as the volume of "dry" plankton per square meter surface have been plotted in Fig. 6 to show the general distribution of animal plankton. For comparison, the highest value of ammonia observed at each station in the upper 80 meters is also shown. While the correlation is not perfect, it is evident that the highest concentrations of ammonia at both periods of observation occur in regions in which the zoöplankton are densely distributed.

This correlation suggests that the animal plankton may be in some way responsible for the appearance of ammonia. While specific observations on plankton animals are lacking, ammonia appears to be the principal nitrogenous waste product of many invertebrate animals (Delauney, 1931). Harvey (1934) has pointed out that the phytoplankton community is continuously being grazed down by the zooplankton. These considerations point to the probability that zoöplankton, through their metabolic products and through their own decay, are an important intermediary in the liberation and distribution of ammonia in sea water.

Summary

1. In the deeper basins of the Gulf of Maine in May ammonia occurred in minimal concentrations at the surface and at all depths below sixty meters; maximal concentrations varying up to 45 mg. N per cubic meter occurred in a definite stratum between 30 and 60 meters.

2. In September the concentration of ammonia was rather uniform at all depths and increased as the distance from the open sea increased, concentrations exceeding 50 mg. N per cubic meter occurring in the western basin.

3. In the tideways of the North and South Channels, ammonia is distributed uniformly with depth in both May and September.

4. In shallow waters its occurrence showed no regularity.

5. The occurrence of ammonia may be correlated in part with the distribution of organic phosphorus compounds, of nitrite, and of

zoöplankton, so as to support the view that its distribution marks the place and the intensity of organic decomposition.

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THE OXYGEN CONSUMPTION OF ARTIFICIALLY ACTIVATED AND FERTILIZED CHÆTOPTERUS EGGS

JEAN BRACHET

(From the Marine Biological Laboratory, Woods Hole, Mass., Biology Department, Princeton University, and the Faculty of Medicine, University of Brussels)

INTRODUCTION

It is well known that unfertilized *Chætopterus* eggs, when they are treated with various physical or chemical agents, undergo a special type of development (F. R. Lillie's (1902, 1906) differentiation without cleavage). Lillie's original observations have been recently confirmed and completed by Pasteels (1934) and by myself (1937). These investigations show that if the unfertilized eggs are treated with 5 per cent isotonic KCl in sea water, they first complete their maturation; immediately after the expulsion of the second polar body, they start undergoing a series of monaster cycles. During each of the cycles, there is an increase in the number of the chromosomes and the appearance of lobulations simulating cleavage. But the furrows fade out quickly and the egg resumes its original shape. There is, therefore, a considerable increase in the size of the egg nucleus and in the number of the chromosomes during the first hours of differentiation without cleavage. Later on, the big single nucleus breaks down and, at the same time, occur processes comparable to gastrulation; in the best cases, unicellular ciliated eggs, resembling somewhat trochophores in their general shape, can be obtained.

It seemed of interest to measure the oxygen consumption of eggs undergoing differentiation without cleavage and to compare it with the respiration of unfertilized eggs: it was hoped that such an investigation would throw some light on the question whether energy is needed for cleavage and differentiation, a problem which has recently been investigated by Tyler (1933, 1936) on other eggs.

A beginning was made last year during a stay in the Zoölogical Laboratory in Naples and a report has been published recently (1937). The results, however, were far from satisfactory because a small percentage only of the eggs could be fertilized, although differentiation without cleavage was easily obtained. Lacking the necessary controls, we could only compare the data obtained for the activated eggs with the results published by Whitaker on the respiration of fertilized eggs in Woods Hole. Obviously, no definite conclusion can be drawn from

such a comparison and it was therefore necessary to reinvestigate the problem.

Material and Methods

The experiments were carried out following the technical details pointed out by Whitaker (1933): one or two ripe females were allowed to shed after the tip of their parapodia had been cut with scissors. The eggs were then washed repeatedly (5 or 6 times) in sea water in order to remove the mucus and a suspension of a concentration varying from 1 : 25 to 1 : 40 was prepared by slightly packing the eggs in a graduated centrifuge-tube and adding the required amount of sea water. Aliquot parts of the suspension (2 cc.) were pipetted into Warburg manometer conical cups (ca. 10 cc. capacity). Fertilization with one drop of diluted sperm or activation with 0.1 cc. of isotonic KCl usually occurred just before attaching the vessels to the manometers. Readings were taken after a 15-minutes equilibration period. The water-bath was kept at 24.8° and the manometers were shaken at a speed of 50 round-trip oscillations per minute with an excursion of 4 cm. That these conditions may be considered as adequate is indicated by the fact that increase in the speed of shaking induced no increase in the oxygen consumption; furthermore, the respiration of unfertilized eggs usually kept constant during 6 or 7 hours and they showed at that time little, if any, cytolysis.

Results

We know since Whitaker's experiments that there is a strong drop in the oxygen consumption of *Chætopterus* eggs at the time of fertilization. My previous experiments from Naples indicated that a similar drop occurs in KCl-activated eggs. In most of those experiments, activation was produced by tipping the KCl contained in the side-arm into the main part of the vessel after the respiration had already been measured during 1 or 2 hours. The results at Naples could be easily confirmed at Woods Hole: while the metabolism of the fertilized eggs dropped to 53 per cent of the initial level (average from 14 experiments in which the percentage of fertilized eggs exceeded 90 per cent), the oxygen uptake of the activated eggs (from the same females) fell to 51 per cent. There is therefore no doubt that, like the increase in respiration which follows fertilization in sea-urchin eggs, the drop observed in the present case is linked to the cytoplasmic changes resulting from activation and not to any special influence exerted by the spermatozoön on the metabolism.

The following graph represents the oxygen consumption of *Chætopterus* eggs during the 7 hours following activation and fertilization.

In this graph, the rate of oxygen consumption has been plotted against time, the initial respiration of the unfertilized eggs being considered as 100 per cent. The individual points on the curves represent average values obtained from 14 experiments.

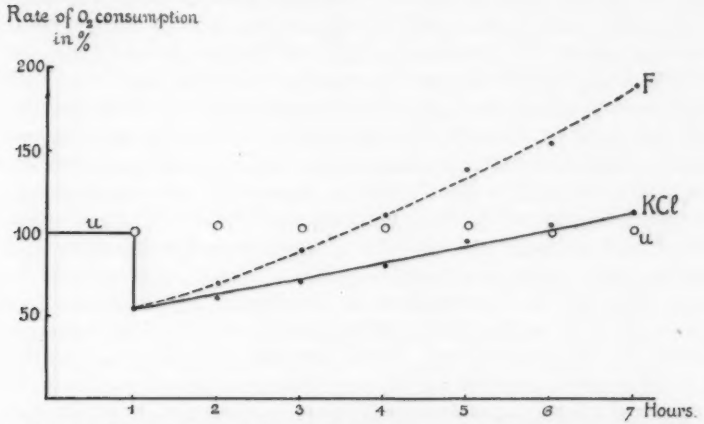

FIG. 1. Oxygen consumption of *Chætopterus* eggs during the seven hours following activation and fertilization. Ordinates: rate of oxygen consumption; abscissæ, hours. The curve *F* represents the oxygen consumption of fertilized eggs; the curve KCl represents that of eggs treated with KCl; and the circles, *u*, represent that for unfertilized eggs.

It is easy to see that, as stated before, the oxygen consumption of the unfertilized eggs (indicated by large circles) remains constant throughout the whole experimental period. It is evident too that respiration increases after the drop in oxygen consumption occurring after fertilization or activation, but obviously this increase is much stronger and faster in the case of the fertilized eggs than it is for the activated eggs: the fertilized eggs reach the initial level after 3½ hours, i.e. at an early blastula stage. The respiration rate increases steadily later on and is almost 80 per cent higher at the end of the experiment than the oxygen uptake of the unfertilized eggs. On the other hand, the activated eggs differentiating without cleavage reach the initial rate only after 6 hours instead of 3½. The fact that the same difference in the respiration of two sets of eggs was found regularly in all the experiments indicates beyond doubt that the observed fact is really significant.

It was possible that the difference might be linked to a depressive effect on the metabolism of the added KCl which was present during the whole experiment. In order to check this possibility, 4 series of

control experiments were run in which the respiration of activated eggs was measured both in presence of KCl in excess as before and after repeated washings (10 times). In other manometers, the oxygen consumption of normal fertilized eggs was compared with the metabolism of similar eggs to which 5 per cent isotonic KCl was added 10 minutes after fertilization. All these control experiments showed that the presence or the absence of KCl in that concentration does not exert any significant effect on the curves. In both cases, the initial respiratory level was reached after 3½ hours in the case of the fertilized eggs and after 6 hours for the activated ones. It seems, however, possible that KCl slightly enhances the absolute O_2 consumption of the eggs. Obviously, the differences observed in the metabolic rate of eggs differentiating without cleavage and of fertilized eggs is somehow linked to their different type of development and not to a depressive effect of KCl on the metabolism.

It was also of some interest to see how the thymonucleic acid synthesis, as a chemical index of the mitotic activity, would compare in fertilized eggs and in eggs differentiating without cleavage. The eggs were therefore taken out of the manometer vessels and preserved in acetone until a sufficient amount of material (about 2 grams) could be collected. The thymonucleic acid content of unfertilized, activated and fertilized eggs was determined by Dische's (1930) colorimetric micromethod. Three estimations could be made. These showed that while the unfertilized eggs contained, as was expected, only traces of thymonucleic acid, the activated eggs had a content in this substance of about 0.35 milligrams per gram wet weight and the fertilized eggs of 1 milligram per gram wet weight. The thymonucleic acid synthesized in 8 hours during differentiation without cleavage amounts thus only to 30 per cent of the amount produced in the fertilized eggs during that period.

Discussion

The curve we obtained for fertilized eggs closely resembles the one published by Whitaker (1933), although its slope is somewhat steeper. In Whitaker's experiments, the unfertilized egg rate is reached by the fertilized eggs in 4½ hours and the increase over that rate at the end of 7 hours of the experiment is only 30 per cent. This difference is very likely due to the higher temperature (24.8° instead of 22°) at which our experiments were carried out: ciliary activity had just begun after 6 hours in Whitaker's case while we noticed swimming blastulae after 4½ hours. It seems therefore probable that conditions similar to those described by Tyler (1936*a*, *b*) in his work on temperature coefficients of developmental processes and cellular respiration prevail

also in *Chætopterus* eggs. It is very probable that the reduced metabolic activity of the eggs differentiating without cleavage is, likewise, somehow linked with their slower development. We have already seen that the control experiments rule out the possibility that the observed difference should result from an inhibition effect of KCl on the eggs' respiration. There is also no doubt that the development of the eggs differentiating without cleavage is slowed down to a considerable extent: for instance, at the end of the experiments the fertilized eggs had turned into actively swimming larvæ while the activated eggs develop cilia only much later. Likewise, the increase of nuclear material, as indicated by the thymonucleic acid estimations, goes on at a much slower rate in the eggs differentiating without cleavage than in the fertilized ones. It is therefore likely that both the reduced oxygen uptake and the slower development are linked together; such a conclusion could support Tyler's opinion that part of the energy available in the egg is needed for the growth and differentiation processes taking place during development. Recent findings by Runnström (1933), J. Brachet (1935), Privolnev (1936), Stefanelli (1937) that the respiration undergoes cyclic changes during mitosis in eggs of different species are also in good agreement with our observations. It must be pointed out, however, that the interpretation of the results is complicated by a special factor; namely, that the activated eggs remain unicellular while the fertilized ones cleave into many cells. It is, of course, by no means impossible that oxidation processes might occur at the surfaces between the different blastomeres. Against such an interpretation, however, two facts can be cited; namely, that there is no direct relationship between the number of the blastomeres and the oxygen uptake of the egg (*cf.* Needham, 1931) and that the respiration of small marine invertebrate eggs is over a wide range independent of the O_2 tension in the surrounding medium.

Summary

1. The oxygen consumption of unfertilized *Chætopterus* eggs drops in the same proportion whether they are fertilized or activated with KCl.

2. The increase in respiratory rate after that initial drop is much faster in fertilized eggs than in activated (differentiating without cleavage) eggs.

3. The lower respiratory activity of the activated eggs is linked to their special type of development.

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ON THE ENERGETICS OF DIFFERENTIATION. VII

Comparison of the Respiratory Rates of Parthenogenetic and Fertilized Urechis Eggs

ALBERT TYLER AND N. H. HOROWITZ

(From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena, California)

The results of these experiments show that the rise in respiratory rate that occurs during development is correlated with cleavage; eggs that fail to cleave after activation show a greatly retarded rise while those that cleave show a rise that is roughly commensurate with their division rate. Inhibiting cleavage with phenylurethane affects the respiratory rate similarly.

Theoretical Part

It has been long been known that the rate of respiration rises during development. This increase in rate is evidently not directly proportional to the increase in the number of cells (*cf.* Needham, 1931). It might, nevertheless, depend upon changes in the egg brought about by cell division, so that when cleavage fails to occur the rise in respiration would be inhibited. We have considered in previous work the dependence of the form-changes on the respiration, the rate of oxygen consumption being taken as a measure of the energy available for the various developmental processes. We consider now the possibility that the developmental changes determine in turn the rate of respiration. If, for example, early cleavage is inhibited in a manner that does not affect the absolute rate of respiration at the particular stage, then we may expect, on this basis, failure of the subsequent rise.

In the early work of Warburg (1910) it has been shown that cleavage could be suppressed in sea urchin eggs by means of phenylurethane without immediately affecting the respiratory rate. However, the question of whether or not the rate would rise later was not investigated. Also it has been shown that after parthenogenetic activation of sea urchin eggs the same increase in rate occurs that is obtained normally upon fertilization (Warburg, 1910; Loeb and Wasteneys, 1913). But here again it would be desirable to know what happens later, especially since the parthenogenetically activated eggs develop much more slowly in general and often stop in early cleavage or even fail to divide.

In *Urechis* artificial activation with a single agent may produce dividing or non-dividing eggs depending on the length of treatment. Diluted sea water (Tyler, 1931) or ammoniacal sea water (Hiraiwa and Kawamura, 1936; Tyler and Bauer, 1937) may be used for this purpose.

Experimental Part

The respiration measurements were made by means of the usual Warburg method using the cylindrical type of vessel previously described (Tyler, 1936) but of 18 to 20 cc. calibration volume. The quantity of eggs present in each vessel was determined at the end of the run from the Kjeldahl nitrogen, and the oxygen consumption is expressed on that basis. The quantity of eggs employed was such as to give readings of 25 to 50 mm. Brodie fluid per hour at the start of the experiment.

The non-dividing and the dividing parthenogenetic eggs were produced simply by treatment with ammoniacal sea water, as previously described. Treatments of 2 to 7 minutes with 0.01 N NH_3 in sea water give 100 per cent activation with normal polar body extrusion but no cleavage. Such eggs go through a series of monaster cycles after polar body formation, but at a slower rate than would correspond to the normal nuclear changes. After about 10 to 15 hours unicellular swimmers may develop similar to the embryos differentiating without cleavage, first described by Lillie (1902). Treatments of 12 to 17 minutes with 0.01 N NH_3 in sea water give 100 per cent activation with as much as 90 to 100 per cent cleavage. Polar body formation is usually interfered with in such eggs, as previously described (Tyler and Bauer, 1937). After 8 to 12 hours swimming embryos including some normal ones may appear.

Non-Cleaving Parthenogenetic Eggs

The results of two sets of experiments with non-cleaving activated eggs are given in Table I, along with the fertilized controls. In both the treatment was for six minutes with 0.01 N NH_3, and insemination of the control was done at the start of the parthenogenetic treatment. Activation and fertilization were 100 per cent in both these experiments. As may be seen in Table I the oxygen consumption of the parthenogenetic eggs during the first hour of measurement is very nearly the same as that of the fertilized eggs. In both experiments the parthenogenetic eggs give slightly higher values, but the difference is no greater here than the difference between the duplicate vessels. During the subsequent hours the rate rises steadily in the case of the fertilized eggs. The parthenogenetic eggs, however, show first a

slight drop (second to fourth hours) followed by an increasing rate. After 8 hours the parthenogenetic eggs attain a rate that is about double their initial respiration, but only about half of that of the fertilized eggs.

The eggs from the vessels were examined at the end of the run. In both experiments the parthenogenetic eggs gave about 2 per cent of unicellular swimmers while the fertilized eggs gave 90 to 98 per cent normal top-swimming trochophores.

Cleaving Parthenogenetic Eggs

The respiration data of two sets of experiments with eggs that divide after ammonia treatment are given in Table II. In both

TABLE I

Oxygen consumption of non-cleaving parthenogenetic eggs. Parthenogenetic treatment = 6 minutes with 0.01 N NH_3 in sea water. Measurements begun at 43 minutes after treatment or insemination in Experiment I and 60 minutes after in Experiment II. Values given as cu. mm. O_2 per hr. per mg. N. Temperature = 22.0° C.

Experiment	I				II		
Hour	Parth.	Parth.	Fert.	Fert.	Parth.	Fert.	Fert.
1st	4.64	4.48	4.38	4.24	4.72	4.53	4.14
2nd	4.40	4.30	4.53	4.38	3.88	5.05	4.49
3rd	4.02	3.99	5.60	5.55	3.83	5.45	5.35
4th	4.52	4.34	7.58	7.53	4.32	7.01	6.65
5th	5.22	5.07	10.01	9.93	5.56	10.10	10.20
6th	6.19	6.02	13.60	13.43	7.40	13.83	13.48
7th	6.86	7.29	16.38	16.31	9.66	18.26	17.45
8th	8.91	8.87	18.66	18.47	10.93	21.64	20.81

experiments it may be seen that the respiration of the parthenogenetic eggs at the start is slightly higher than that of the fertilized controls. A third set not presented in the table shows the same initial difference, and also agrees very well throughout the run. The difference here is somewhat greater than was manifested in the experiments with the non-cleaving parthenogenetic eggs. It may be pointed out that the ammonia treatment is considerably longer here (13 to 17 minutes) and this may account for the higher initial rate.

The subsequent readings give values that are fairly constant for the parthenogenetic eggs up to the fifth hour, while the fertilized eggs rise as usual during this time. Following this the rate rises and at the eighth hour the respiration is about half of the control rate. It is very nearly the same at that time as is obtained with the non-cleaving

parthenogenetic eggs (Table I). However, differences appear later as will be shown below.

The cleavage of these parthenogenetic eggs is very slow compared with the fertilized controls. In Experiment I, at 2 hours after treatment, there were 43 per cent cleaved of which 15 per cent were in two cells, 22 in three and 6 in four. The fertilized controls were 100 per cent in four at this time. An hour later there was 75 per cent cleavage. At the end of the run (11 hours after treatment) the eggs from the vessels were 90 per cent cleaved and 30 per cent were swimmers.

In Experiment II, 60 per cent of the parthenogenetic eggs were divided at two hours after treatment, the distribution being 23 per cent

TABLE II

Oxygen consumption of cleaving parthenogenetic eggs. Parthenogenetic treatment = 13 minutes (Experiment I) and 17 minutes (Experiment II) with 0.01 N NH_3 in sea water. Measurements began at 74 minutes after treatment or insemination in Experiment I and 53 minutes after in Experiment II. Values given as cu. mm. O_2 per hour per mg. N. Temperature = 22° C.

Experiment	I				II			
Hour	Parth.	Parth.	Fert.	Fert.	Parth.	Parth.	Fert.	Unfert.
1st	5.88	5.81	4.48	4.64	5.69	5.70	4.51	3.69
2nd	5.45	5.69	4.92	4.82	5.40	5.48	4.60	3.90
3rd	5.20	5.25	5.63	5.80	5.51	5.34	5.59	3.37
4th	5.36	5.36	7.03	6.96	5.85	5.70	6.94	——
5th	5.63	5.25	10.28	10.12	5.91	5.48	9.07	3.29
6th	5.99	5.92	14.05	13.80	7.01	6.86	13.17	3.37
7th	7.55	7.25	17.46	17.83	8.04	8.02	16.27	
8th	8.62	8.04	20.90	21.14	10.25	10.18	20.06	
9th	9.71	9.49	24.50	24.45				
10th	10.65	10.95	——	28.55				

in two cells, 30 in three and 7 in four. The fertilized controls were all in four cells at the time. At the end of the run (10 hours) the parthenogenetic eggs from the vessels were 95 per cent cleaved of which 5 per cent were swimmers, while the fertilized eggs gave 90 per cent swimmers.

Since the parthenogenetic eggs do not divide at all synchronously and since many may stop after one or more cleavages, the distribution becomes quite complicated. It is clear, however, that cleavage and the development of cilia are retarded.

Later Stages

Measurements on the later stages were made by culturing the eggs in dishes and washing the embryos before transfer to the vessels.

This is to avoid possible effects of bacterial growth or other changes produced in the vessels during a prolonged run. In Table III the results of one set of experiments are given. Cleaving and non-cleaving parthenogenetic eggs were prepared from the same batch along with the fertilized controls, and allowed to develop for 11 hours at room temperature (18.2° C.) before transfer to the vessels. In the fertilized lot there were more than 95 per cent top-swimming young trochophores, and only the top swimmers were transferred to the vessels. In the non-cleaving parthenogenetic lot (Parth. 5) there were no swimmers at that time. In the cleaving parthenogenetic lot (Parth. 17) 95 per cent had divided and practically all of them were bottom-swimmers.

TABLE III

Oxygen consumption of late stages of non-cleaving and cleaving parthenogenetic eggs. Parthenogenetic treatment = 5 minutes (Parth. 5) and 17 minutes (Parth. 17) with 0.01 N NH_3 in sea water. Eggs cultured for 11 hours at 18.2° before measurements were begun. Values given as cu. mm. O_2 per hour per mg. N. Temperature = 22°.

Hour	Fert.	Fert.	Parth. 5	Parth. 5	Parth. 17	Parth. 17
1st	21.80	22.09	12.31	12.17	11.64	11.69
2nd	25.45	25.91	12.54	12.42	19.03	18.45
3rd	29.38	30.48	15.11	14.90	18.74	18.96
4th	32.39	33.32	15.01	14.65	20.75	20.26
5th	36.17	36.18	14.44	14.15	22.60	22.35
6th	39.94	40.48	15.76	15.72	24.76	24.98
7th	43.31	45.70	18.98	18.75	27.70	27.95
8th	46.50	47.70	22.18	22.73	31.73	32.67

As may be seen from Table III the two types of parthenogenetic eggs respire at very nearly the same rate at the start of the experiment. The rate is a little more than half of the fertilized rate at this time. The rate rises with time, but more rapidly in the case of the dividing eggs. At the end of the eight-hour period they are respiring at almost one and a half times the rate of the non-cleaving eggs, but still only two-thirds that of the fertilized controls. Examination of the eggs from the vessels at the end of the run showed 20 to 30 per cent bottom-swimmers (unicellular) in the case of the non-cleaving eggs (Parth. 5); 100 per cent swimmers of which 30 to 40 per cent were top-swimmers in the case of the cleaving eggs (Parth. 17); and 100 per cent top-swimmers in the case of the fertilized controls.

Fertilized Eggs in Phenylurethane

With the proper concentration of phenylurethane, cleavage may be suppressed without the initial respiratory rate being affected, as

Warburg (1910) showed on sea urchin eggs. The experiments were repeated for the purpose of covering a longer period. The results of such an experiment with *Urechis* eggs are presented in Table IV. The eggs were placed in the solution at 45 minutes after insemination, and the measurements begun 40 minutes later. As the figures in Table IV show, the initial rate of respiration is the same as in the untreated controls. The rate then rises in both, but more slowly in the treated eggs. At the end of the run their respiration is less than three-fourths of the control rate.

It is evident here that the failure of cytoplasmic cleavage is accompanied by a slower rise in respiratory rate. The rate does, however, actually rise. It may be pointed out in this connection that nuclear division goes on in the treated eggs but at a retarded rate.

TABLE IV

Oxygen consumption of phenylurethane-treated eggs. Eggs placed in 5×10^{-4} N phenylurethane after appearance of second polar body (45 minutes after insemination). Measurements begun at 85 minutes after insemination and 40 minutes after immersion in phenylurethane. Values given as cu. mm. O_2 per hour per mg. N. Temperature = 22.0° C.

Hour	Control	Control	Phenylur.	Phenylur.
1st	4.61	4.64	4.86	5.04
2nd	4.94	4.92	4.69	4.83
3rd	5.66	5.48	5.40	6.71
4th	6.99	7.05	6.72	5.13
5th	9.84	10.02	9.16	10.84
6th	13.34	13.54	10.80	9.26
7th	16.30	16.40	12.30	14.01
8th	18.83	18.79	13.68	14.01
9th	21.61	21.33	15.43	15.98

Runnström (1928) investigated the action of phenyl- and ethylurethane on the respiration of sea urchin eggs. The concentrations that he used gave a depression of the initial rate; nevertheless a distinct rise is manifested during the three-hour period of the experiments. He notes too that nuclear division proceeds although the respiration during the first hour may be only 35 per cent of that of the control.

DISCUSSION

The curves of Fig. 1 summarize the results. It is readily seen that the parthenogenetic eggs, although starting out at about the same rate as the fertilized eggs, do not give as rapid a rise with time. The two types of parthenogenetic eggs give approximately the same values during the early stages, but later the dividing eggs manifest a

more rapid rise. If only the early period were considered this might be taken to mean that the rise in respiratory rate is not at all correlated with cleavage. However, as pointed out above, the cleavage parthenogenetic eggs divide at a retarded rate. Also, it is evident that in normal fertilized eggs no appreciable rise occurs during the early cleavage. It is therefore fairly safe to conclude that the rise in rate is linked with cleavage. But that it is not merely a matter of cytoplasmic cleavage is clear from the fact that in the non-cleaving parthenogenetic eggs and the phenylurethane-treated eggs the respiratory rate does rise with time. Here, as we have seen, nuclear division goes on and it is probably with this factor that the rise is connected.

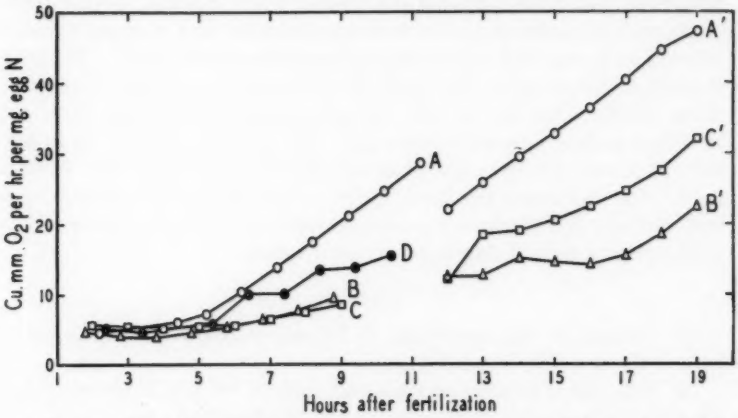

FIG. 1. Rate of oxygen consumption of *Urechis* eggs. Curves *A* and *A'*; fertilized eggs. *B* and *B'*; non-cleaving parthenogenetic eggs. *C* and *C'*; cleaving parthenogenetic eggs. *D*; phenylurethane-treated eggs. Temperature 22° C. Values are averages from all of the data. *A'*, *B'* and *C'* are not direct continuations of *A*, *B*, and *C* since the eggs had been cultured at 18.2° C. for 11 hours.

Similar experiments have recently been independently performed by Brachet (1938) on *Chætopterus* eggs that "differentiate without cleavage" and he has kindly allowed us to examine his manuscript. The results with *Chætopterus* agree very well with those presented here. In addition Brachet has investigated the nucleic acid content and finds it to be much lower in the parthenogenetic (non-cleaving) eggs than in the fertilized controls.

In experiments of this type it is, of course, important to rule out possible direct effects of the chemical agent employed. It is reasonable to assume that there has been no direct effect when the initial rate is unaltered. Such is the case in the experiments reported here with

the exception of the cleaving parthenogenetic eggs (Table II) which showed a significantly high initial respiratory rate. This is very likely due to the ammonia treatment and from the subsequent values we might assume that the effect passes off. In any event, the difference would have to be greater and in the opposite direction to seriously affect the conclusion.

As was pointed out in the introduction, we might expect no rise in respiration if cleavage were blocked by some means that does not alter the absolute respiratory rate at the particular stage. But we are not dealing here with such ideal cases. In these experiments cytoplasmic division fails (non-cleaving parthenogenetic eggs) or is prevented (phenylurethane-treated eggs) while nuclear division proceeds at a retarded rate; or both cytoplasmic and nuclear division proceed at a retarded rate (cleaving parthenogenetic eggs). It is not surprising, therefore, to find that the respiratory rate does increase in these cases. That the parthenogenetic show a slower rise than the fertilized eggs is consistent with their slower development, as is also the difference between the phenylurethane-treated and the control eggs. The difference between the two types of parthenogenetic eggs may likewise be interpreted as due to differences in the rate at which comparable stages of development are reached.

Summary

1. Artificially activated eggs of *Urechis* respire at the same initial rate as do normally fertilized eggs.

2. The rate of respiration rises with time in the artificially activated eggs, but at a much slower rate than in the fertilized eggs.

3. The increase in respiratory rate with time is greater with cleaving than with non-cleaving parthenogenetic eggs.

4. Fertilized eggs in which cytoplasmic cleavage is inhibited and nuclear division retarded by means of phenylurethane give a retarded rise in respiratory rate, although the initial rate is the same as the control rate.

5. It is concluded that the delayed rise in respiration is linked with the slower development in all these cases.

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EXPERIMENTS ON LIGIA IN BERMUDA

V. Further Effects of Salts and of Heavy Sea Water

T. CUNLIFFE BARNES

(From the Osborn Zoölogical Laboratory, Yale University, and the Bermuda Biological Station)

Four previous papers of this series (Barnes, 1932, 1934, 1935, 1936) have described aspects of the behavior, salt requirements and thermal range of *Ligia baudiniana*, an interesting isopod which is invading the land through the intertidal zone. The present report deals with further experiments on the capillary mechanism conveying sea water to the gills, reactions to filter paper saturated with diluted sea water, the protective action of Ca in hypotonic sea water and the combined effect of high temperature and heavy water.

Habits

It was reported previously that the release of young from the brood pouch was observed only in specimens kept submerged in sea water, but during the past summer this phenomenon occurred in a few cases in females in air over filter paper moistened with sea water. On the other hand, the molting process has occurred only in air. No adult specimens have been taken in the sea, but in rare cases an isopod will enter sea water in a terrarium to feed.

Ligia is provided with a capillary mechanism for keeping the gills moist without entering the sea. The first paper of this series stated that the uropods and spines are lowered into the water or onto a water film and the sea water then rises by this capillary path to the gills. However, Mr. M. D. Burkenroad has drawn my attention to a more important capillary conduit by which the water rises between the sixth and seventh legs to the gills and is then propelled by the gills *down* the uropods and spines (Fig. 1, *A*). The sixth and seventh leg on one or both sides may be used. Frequently the animal slides one leg over the other alternately at the start. The drainage down the uropods was described in a former paper (Barnes, 1935) in experiments in which sea water was dropped on the animals. The flow was followed in the present experiments by the addition of stains, fine particles or bubbles to the sea water. If the uropods are lowered when the last pair of legs are not drawn together, the first movement of the water is up the capillary conduit of the uropods as originally described (which

may be observed by the movement of particles in the sea water). When the isopod is totally immersed, the currents produced by the beating of the gills in the surrounding sea water (indicated by the movement of particles) follow the same direction as the capillary circuit involving the rise of water between the last pair of legs (Fig. 1, B).

The sixth or seventh leg was removed in some specimens to modify the method of obtaining water. In the absence of the seventh leg a specimen standing on moist filter paper usually takes up the position

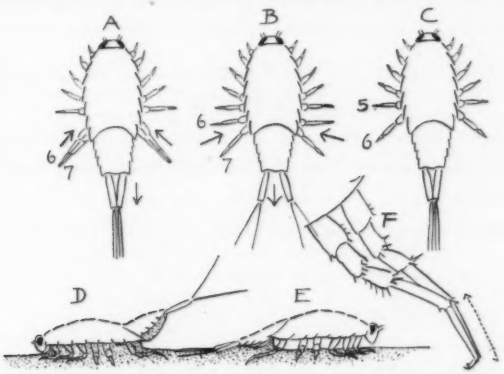

FIG. 1. Methods of moistening the gills in *Ligia*. (In these sketches the antennae and body segmentation are omitted.)

A. Specimen in air on filter paper saturated with distilled water. Water rises between the sixth and seventh leg by capillarity and is drained down between the uropods as indicated by arrows.

B. Specimen immersed in sea water. The arrows indicate the currents maintained by the beating of the gills.

C. Specimen with seventh leg removed standing on filter paper saturated with sea water. Sixth leg and uropods are in position for capillary circuit.

D. Side view of specimen with sixth leg removed standing on film of sea water (stippled).

E. Side view of specimen with sixth leg removed with abdomen lowered in film of sea water.

F. Sixth and seventh legs permanently fused along segment indicated by dotted line (found on left side of one specimen). These partially fused legs functioned normally as a capillary channel.

for the capillary circuit via the legs (Fig. 1, *C*). It feels about frantically with the sixth leg for the missing seventh leg to complete the capillary channel. No attempt is made to use the fifth leg. Similarly if the sixth leg is removed, the conduit is impossible. Some specimens "squat" on the water film and the water rises by capillarity on the whole undersurface of the body and gills (Fig. 1, *D*). Other specimens with the sixth leg missing lower the abdomen and uropods and thus

secure water by capillarity (Fig. 1, *E*). It is clear that there are several methods of wetting the gills. Indeed, many specimens are found which have lost the uropods and spines. These simply lower the abdomen on a damp substratum.

An interesting specimen was found in which the sixth and seventh leg were permanently fixed in the capillarity posture owing to the fusion of the last segments (Fig. 1, *F*). While walking, the double leg moved alternately with the seventh leg on the opposite side, but sometimes dragged. The joined legs functioned normally as a channel above the point of fusion.

Reaction to Filter Paper Saturated with Diluted Sea Water

Large filter papers (diameter 25″) were cut in two, one half saturated with distilled water, the other with diluted sea water and placed

Table I

Reaction of Ligia to Filter Paper Saturated with Diluted Sea Water
(The animals were tested in groups of four)

Treatment of each half of paper	Total number of isopods found on each half	Ratio
Sea water *vs.* distilled water	69 : 123	1 : 1.78
75 per cent sea water *vs.* distilled water	27 : 73	1 : 2.74
50 per cent sea water *vs.* distilled water	93 : 96	1 : 1.03
25 per cent sea water *vs.* distilled water	109 : 73	1 : 4.9
10 per cent sea water *vs.* distilled water	27 : 21	1.28 : 1

in a covered flat dish in a photographic darkroom having a light directly above the center of the dish. Four isopods (previously kept on seaweed moistened with sea water) were placed in the dish and their distribution on the two halves was observed at ten to fifteen-minute intervals over a period of one to two hours. The dish was rotated 90° after each reading to eliminate any unsuspected source of orientation. The animals showed a distinct tendency to collect on filter paper saturated with distilled water when the other half was moistened with sea water (Table I, and Barnes, 1935). It was found that this aversion was also shown to 75 per cent sea water, but not to dilutions of 50 per cent and below (Table I).

The tendency to avoid sea water was most pronounced during the first observations in a given experiment and after an hour or more, when the paper containing distilled water was becoming dry, most specimens collected on the sea water side. Thus the ratio of specimens on the sea water side to those on the distilled water side was 1 : 3.3

for all the first readings and 2.3 : 1 for the last observation made after an average time interval of one and one half hours.

The Protective Action of Calcium in Hypotonic Sea Water

Ligia survives for only about seven hours in 100 cc. of 25 per cent sea water, but as with many other forms, the addition of calcium protects the organism from hypotonic media, apparently by decreasing permeability. As in all the experiments with solutions, individual specimens were tested in 100 cc. of solution in finger bowls. As will be seen from Table II, there is a threshold for the Ca effect (at about

Table II

Longevity of Ligia in 25 per cent Sea Water Containing Added $CaCl_2$

cc. $\frac{1}{2}$ M $CaCl_2$ added to 1 liter 25 per cent sea water	Average longevity	Maximum longevity	Coefficients of variation	Number of specimens
	hours	*hours*		
0	7 ± 0.33	15	4.7	33
2	6.5 ± 0.20	11	3.0	30
5	7.6 ± 0.37	17	4.8	58
12	24.6 ± 3.66	119	14.8	30
15	24.1 ± 3.70	144	15.3	20
20	51.5 ± 7.98	120	15.5	20
25	37.8 ± 5.09	168	13.5	30
50	38.5 ± 3.85	180	10.0	40

12 cc. $\frac{5}{8}$ M $CaCl_2$ added to a liter of 25 per cent sea water). It is also apparent that the addition of Ca above a critical quantity (20 cc. $\frac{5}{8}$ M $CaCl_2$ added to a liter of 25 per cent sea water) has little additional protective action. It is interesting to note (see Fig. 2) that the average survival in calcified 25 per cent sea water approaches the average longevity in 100 cc. of natural sea water.

The Reversibility of the Calcium Effect

Experiments were carried out to see if the protective action of calcium involves an irreversible chemical process. If so, preliminary treatment in solutions of high calcium content should lengthen the survival of isopods subsequently immersed in hypotonic sea water. However, it was found that preliminary immersion in calcified sea water from half an hour to over a day had little if any effect on the subsequent longevity of the treated specimens in 25 per cent sea water (see Table III). Likewise, preliminary treatment with sea water having a high content of sodium has no significant effect on subsequent action of hypotonic sea water. In all these tests many specimens

died during the preliminary treatment so that those tested in the 25 per cent sea water represented a selected population capable of withstanding the submerged state. It must be remembered, however, that the survival of *Ligia* in any liquid medium is limited by unknown factors so that these selected specimens were already weakened by submersion. That these two factors balanced each other is indicated by the similarity of the average longevity in 25 per cent sea water of untreated and treated specimens. Thus the 152 isopods exposed to

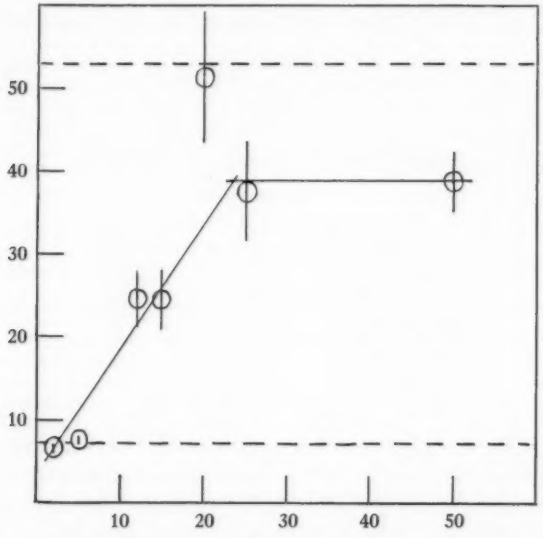

FIG. 2. Length of life of *Ligia* in 100 cc. of 25 per cent sea water with increasing amounts of $CaCl_2$.

Ordinates, average life in hours. Abscissæ, cc. of $\frac{1}{8}$ M $CaCl_2$ added to 1,000 cc. of 25 per cent sea water. The graph represents 228 tests (see Table II). Lower dotted line is the expected survival in 25 per cent sea water. Upper dotted line is the average survival in natural sea water. The vertical lines are proportional to the probable errors. The sea water average represents 265 specimens and the 25 per cent sea water average, 63 specimens. These are cumulative totals for five seasons' work. In all cases an individual specimen was tested in 100 cc. of solution.

previous solutions survived 8.9 hours in 25 per cent sea water compared to the usual average of about seven hours in this medium for freshly collected individuals. In all the tests the specimens were washed by rapid immersion in distilled water after treatment in the calcium solutions, but this had no effect on the subsequent survival in 25 per cent sea water. Thus controls treated with ordinary sea water and

then washed in distilled water lived the usual interval of about 7 hours in 25 per cent sea water.

Experiments in which the isopods were tested in distilled water after treatment with $CaCl_2$ give similar results (see Table IV). After exposure to $\frac{5}{8}$ M $CaCl_2$ the average survival was three hours in distilled water compared to 2.8 hours for untreated specimens.

The Combined Effects of High Temperature and Heavy Water

It was previously shown that *Ligia* succumbs rapidly in a small volume of sea water at 38° C. The following experiments were performed to ascertain if sea water containing 99 per cent heavy water would protect the organism against heat death or else potentiate with high temperature by reducing the survival time. In each case 5 cc.

Table III

Longevity of Ligia in 25 per cent Sea Water after Treatment in Calcified Sea Water

Initial solution	Average length of treatment	Subsequent average longevity in 25 per cent sea water	Maximum longevity	Coefficients of variation	Number of specimens
	hours	*hours*	*hours*		
Sea water	12.2	6.2±0.30	13.5	4.8	34
50 cc. $\frac{5}{8}$ M $CaCl_2$ plus 950 cc. sea water	31.5	5.5±0.32	6.5	5.8	13
100 cc. $\frac{5}{8}$ M $CaCl_2$ plus 900 cc. sea water	21.1	6.5±0.33	9	5.0	22
200 cc. $\frac{5}{8}$ M $CaCl_2$ plus 800 cc. sea water	17.6	9.2±0.38	17	4.1	49
500 cc. $\frac{5}{8}$ M $CaCl_2$ plus 500 cc. sea water	0.5	10.5±0.39	18	3.7	40
400 cc. $\frac{5}{8}$ M NaCl plus 600 cc. sea water	25.3	9.4±0.79	25	8.4	28

of sea water were evaporated and the salts redissolved in ordinary distilled water or in 99 per cent heavy water. It was found that death ensued twice as rapidly in heavy sea water at 38° C. The survival times in 5-cc. samples were 23 minutes for H_2O sea water and eleven minutes for D_2O sea water (see Table V). Owing to the scarcity of heavy water, several specimens were tested in the same sample, but no appreciable difference was observed in the survival times of the first and last isopods treated.

Discussion

The tendency of the isopods to collect on filter paper treated with distilled water rather than on sea water paper is of interest in connection with the animals' aversion for the sea. The reaction appears to

TABLE IV

Longevity of Ligia in Distilled Water after Treatment with Calcium

Initial solution	Average length of treatment	Subsequent longevity in distilled water	Maximum longevity	Coefficients of variation	Number of specimens
	minutes	*hours*	*hours*		
None	0	2.8±0.25	5.2	8.9	22
500 cc. $\frac{2}{3}$ M $CaCl_2$ plus 500 cc. sea water	33	4.5±0.15	6	3.3	20
$\frac{2}{3}$ M $CaCl_2$	12	3±0.20	4	6.7	12

be determined by the salt content of sea water since dilution of 50 per cent destroys the effect. It is possible that the salts on the paper stimulate the isopods to greater movement which would cause them to collect on the salt-free side. Gunn (1937) has described a hygrokinetic effect in *Porcellio scaber* whereby the greater activity of animals in dry air causes them to collect in moist locations, but this mechanism is probably not responsible for the reaction of *Ligia* described above. As was shown in a previous paper (Barnes, 1935), specimens will collect on dry filter paper when the other side of the dish contains filter paper moistened with distilled water. This refers to moist specimens. It is not known what part the flushing mechanism of the gills plays in

TABLE V

Longevity of Ligia in 5 cc. of Ordinary and Heavy Sea Water at High Temperature

Medium	Temperature	Average longevity	Maximum longevity	Coefficients of variation	Number of specimens
	° C.	*seconds*	*seconds*		
Sea water	38	1383±45.1	2005	3.26	27
Sea water containing 99 per cent D_2O	38	660±57.0	1140	8.63	11

these reactions. Bateman (1933) found that salts become slightly concentrated in the blood of specimens kept in air and it is possible that flushing the gills, especially with distilled water, enables the animal to get rid of salts concentrated by evaporation. On the other hand, specimens which have been immersed in distilled water also show the aversion for paper soaked in sea water (Barnes, 1935).

The well-known action of Ca as a factor enabling organisms to withstand hypotonic solutions (for reference *cf.* Barnes, 1934) is strikingly illustrated by *Ligia*, which survives almost as long in 100 cc. of 25 per cent sea water of approximately 0.015 M $CaCl_2$ content as in 100 per cent of natural sea water. To the list of favorable artificial

sea water solutions for *Ligia* described in previous papers may be added this new hypotonic mixture which is equivalent to sea water in which the Na, K and Mg content has been reduced to one fourth and the Ca content raised slightly. However, the *maximum* longevity in this medium is far short of the 297 hours observed in natural sea water. The very long survival of occasional specimens in sea water suggests that other factors besides salt effects are involved, such as the amount of food in the gut, the nature of the previous environment, the oxygen content, or the necessity for molting (which apparently does not occur in sea water). The high summer temperature of approximately 27° C. must also be considered.

The failure of preliminary immersion in sea water rich in calcium to protect the organism against subsequent exposure to hypotonic sea water indicates that Ca forms a loose, rapidly reversible combination with material in the plasma membrane. The existence of a threshold concentration and of a limited range in which the effect of Ca is proportional to the concentration suggests that a surface reaction is involved.

The rapid lethal action of 5 cc. of sea water at 38°, in which the ordinary water has been replaced by heavy water, was an unexpected result. The lower energy content of heavy water, which under certain conditions might be expected to protect an organism from high temperatures, was overbalanced by the well known toxic action of high concentrations of deuterium. Barbour (1937) has recently discussed the toxicology of heavy water. In larger volumes of sea water or even fresh water *Ligia* withstands high temperatures for a much longer period, probably on account of the greater quantity of oxygen present. The 5-cc. samples used in the tests were exposed to the air in a Petri dish so that the oxygen content of both heavy and ordinary sea water was probably the same.

Summary

1. When presented with a "choice" between filter paper moistened with sea water or with distilled water, freshly caught specimens of *Ligia* tend to collect on the latter. Dilution of the sea water destroys this effect.

2. The survival of *Ligia* immersed in 25 per cent sea water with added calcium approaches the longevity in natural sea water.

3. Preliminary exposure of solutions of high calcium content does not protect *Ligia* from subsequent immersion in hypotonic sea water.

4. The lethal action of 5 cc. of sea water at 38° C. is enhanced by 99 per cent heavy water.

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SOME ASPECTS OF NORMAL DEVELOPMENT IN THE COLONIAL CILIATE ZOÖTHAMNIUM ALTERNANS

F. M. SUMMERS

(From the Marine Biological Laboratory, Woods Hole, and Bard College, Columbia University)

INTRODUCTION

This is the first of a series of inquiries into some of the problems of organization and regulation in a colonial protozoan of a rather special type, *Zoöthamnium alternans*, whose cells collectively possess in some degree many of the attributes of an individual organism. In order to fix some standard by which to judge experimental results it was first necessary to review in detail the characteristic features of normal development in a large number of cases.

Although Claparède and Lachmann (1858) formulated a scheme of notation for cell lineage in this species, Fauré-Fremiet (1930) was the first to make a comprehensive study of its development. His primary concern was the demonstration of cytological mechanisms by which potentialities for growth and ciliospore formation were assorted to cells in characteristic positions on the colony. According to his conclusions this "cytological" factor determines the general pattern of development. His second objective, a study of time as a limiting growth factor, has no immediate bearing on the subject to be treated here. The quantitative aspects and time relations in development are to be incorporated in a subsequent report.

The data presented here augment in considerable detail the previous studies of *Zoöthamnium alternans*, particularly with regard to the features of development which have an important bearing on the problems of regulation now being investigated.

The work was done at the Marine Biological Laboratory, Woods Hole, Massachusetts, during the summer months of 1935–6.

It is a privilege to acknowledge assistance from those whose friendly interest has sustained the author's enthusiasm over many of the dull periods of routine work.

MATERIAL

The basal portions of mature hydroids (*Pennaria* and *Tubularia*) and Bryozoa (*Bugula*) from many localities in Woods Hole harbor great numbers of attached colonies of *Zoöthamnium alternans*. The

hydroid and bryozoan colonies are clean and free from the ciliates until about June 15. From then on throughout the summer the protozoa are very abundant, although their condition varies with particular habitats and with the water temperature. Grave (1933) found that the hydroids of this region are particularly healthy and free from bacterial growths, debris, etc. for the early spring months but that in mid-summer the contaminating organisms affect them adversely. This applies equally to the associated protozoa, for those obtainable in late July and August are commonly infested with parasites of several classes. The most annoying and destructive organisms are bacteria, filamentous algæ, and an intra-cellular parasite which has been identified as a suctorian (*Acineta*) by Fauré-Fremiet (1930); others of less consequence are small crustacea, diatoms, and other ciliates, e.g. *Amphileptus* and *Cothurnia*.

Methods

The procedure for maintaining selected colonies in aquaria is an elaboration of the technique employed by Fauré-Fremiet (1930) for his study of growth in *Zoöthamnium alternans*. Hydroids bearing the protozoa were cleaned, washed, and packed into large glass vessels of running sea water. Ruled and numbered slides were placed face down on the infected hydroid material and left for about 12 hours. They were then removed and washed by passing under a stream of running water. The precautions taken at these stages to remove adhering scum, debris, algæ, other protozoa, and minute crustacea, determined to a great extent the age to which the colonies could be grown.

Some of the mature asexual migratory cells, the ciliospores (Wesenberg-Lund, 1925), liberated from the "wild" colonies during the 12-hour interval, attached themselves to the ruled surface of the numbered slides. Slides were examined in Petri dishes, the positions of the recently affixed ciliospores recorded, and then transferred from the Petri dish to correspondingly numbered slots (vertical) in wooden racks, immersed in a 15-gallon aquarium. Cultural conditions were very much improved by the use of filtered sea water. Two 2-liter aspirator bottles packed with sand and stoppered with glass wool proved to be adequate for the purpose. When cleaned daily their combined outputs averaged about 50 gallons per hour.

Routine counts were made with a 4 mm. dry objective. When optical sections of the barely immersed colonies were not clear, the water level in the Petri dish was adjusted by a slight turn of the dish on the slanted microscope stage until the surface film flattened the

colony. This was done as a last resort, for contact with the surface film elicited rapid contractile responses, frequently resulting in the loss of important zoöids.

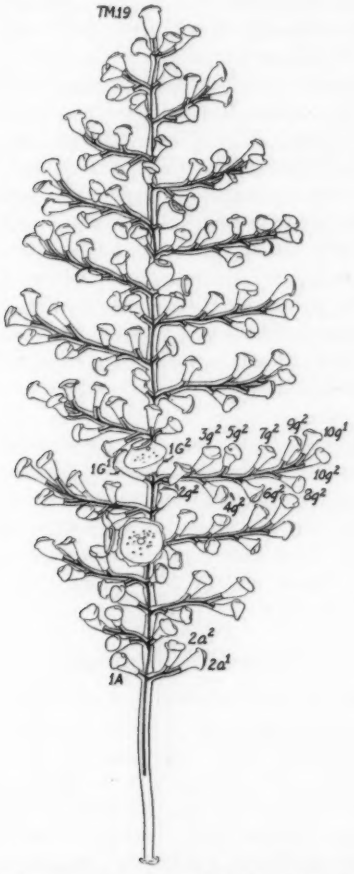

FIG. 1. A relatively mature colony of *Zoöthamnium alternans* showing the characteristic arrangement of the zoöids and branches. Axial microzoöids on branches *E*, *G*, *L*, and *N* are differentiating into ciliospores; one of them at *E* is in an advanced stage of metamorphosis, almost ready to break away from the parent colony. Approximately × 100.

GENERAL FORM OF THE MATURE COLONY

The tapering frond-like configuration of the older expanded colonies is shown in Fig. 1. Its branches project obliquely from the per-

pendicular axis. They lie in the same plane but alternate on right and left sides at successive nodes. The zoöids of the branches occur singly for the most part, likewise alternating in position along the branch axis. While the branches of this species usually consist only of a single axis, in rare cases the zoöids may divide one or more times to produce branches of the second order.

The expanded colony is essentially asymmetrical, i.e. characterized by three heteropolar axes. The primary axis is firmly cemented to the substrate at the basal end and bears the actively dividing terminal macrozoöid (*TM.*) at the apex. The lateral axis is heteropolar by virtue of the alternating branches, each one terminating in a single cell, the dominant branch zoöid. "Anterior" and "posterior" surfaces (Fauré-Fremiet, 1930) are defined by the curvature of the branches as well as by the position of the zoöids: the branches curve anteriorly while the zoöids are inclined posteriorly (toward the convex surface). A sample count showed that the first branch occurred nine times on the left side to four on the right. Both dexio- and leiotropic colonies were reported in another species, *Zoöthamnium arbuscula*, by Furssenko (1929).

Beginning at a point about mid-way between the basal (attached) end of the stalk and the first branch, a heavy contractile central cordon, or "neuro-muscular" cord, within the stalk substance ramifies throughout the colony (Fig. 1). Its branches terminate distally at the basal ends of the zoöids. It is thus continuous from branch to branch and from cell to cell. More will be said about the importance of this structure in a later section.

Normal Development

The scheme used to designate the lineage of the zoöids is a modified form of Fauré-Fremiet's adaptation of the original rule formulated by Claparède and Lachmann (1858–60), who were the first to note development in this species according to a definitely determined pattern.

The mode of propagation in *Zoöthamnium alternans* is almost exclusively asexual. Axillary branch zoöids at various levels in larger colonies are transformed into migratory ciliospores. These liberated motile individuals are comparatively large cells, metamorphosed zoöids, whose sub-conical bodies flatten antero-posteriorly into cells having a thickened bi-convex lens-like appearance. Their rapid swimming or creeping movements are effected by means of a heavy equatorial girdlet of cilia. The oral disc and peristomal cilia are retracted and probably undergo reorganization during the motile phase.

After a migratory existence of several hours duration the ciliospores become relatively quiescent, hovering about within a limited radius with aboral end in contact with the substrate, finally fixing themselves to the surface by means of a scopula. The secretion of the stalk begins; the girdlet of cilia becomes inactive and disappears as the peduncle elongates. The lens-shaped ciliospore then begins to assume the appearance of a very large sub-conical zoöid with retracted adoral zone. The first section of the peduncle secreted is a fairly heavy, non-contractile cylinder with thickened cuticle and homogeneous hyaline medullary substance. At some point between 200–300μ the secretory activity of the ciliospore is altered: the continuity of the secretory process is interrupted, the basal end of the cell increases in diameter, and the formation of the neuro-muscular cord begins. This node marks the lower limit of stalk contractility. The contractile cord is an integral part of the stalk structure from this point onward.

The division of the initial individual occurs approximately 15 hours after attachment. The first division furrow defines the median plane of the future colony. Succeeding furrows bear a definite and constant relation to the first, in such a manner that the daughter cells occur first on one side of the mid-line then on the other. The two daughter cells resulting from the first division are always markedly unequal in size (Fig. 2). The large daughter remains apical (terminal) in position.

FIG. 2. Developing colony at the 2-cell stage. Drawn from a living specimen in which the macronuclei were faintly visible. × 250.

In the current terminology it is designated as the terminal macrozoöid No. 1. The small lateral daughter, the first cell of the first branch, is characterized as the median microzoöid *A*.

Dichotomous branching of the stalk occurs when the secretory basal portion of the cell constricts during the fission process. With the onset of secretory activity (i.e. stalk formation) the sister cells gradually move away from the point of origin. Material secreted by the terminal macrozoöid prolongs the axial stalk. The branch cell produces a somewhat smaller stalk extending laterally from the junction.

The apical cell (*TM.* No. 1) divides again at the second node in approximately 8 hours, producing an apical cell of the second generation (*TM.* No. 2) and a median microzoöid *B*. This time, however, the branch cell lies on the opposite side of the axis from the previously produced microzoöid *A*. Repeated divisions of the apical cell extend the principal axis of the colony, each time similarly producing a terminal macrozoöid of the succeeding generation (3, 4, 5, . . .) and an alternating branch zoöid of a higher level (*C*, *D*, *E*, . . .). The position of successive branch microzoöids is strictly alternative. Colonies with as many as 25 branches were frequently observed on the culture slides but it is doubtful that this represents maximum development. One colony produced 33 branches before succumbing to external parasites.

Following the first division of the apical cell there is a gradual reduction in the size inequality of the resulting daughters until, after 8 to 10 generations of axial development, or in some cases even fewer, their volumes immediately after a mitotic period are approximately equal. For experimental purposes it is essential to distinguish between recently formed daughters of later generations along the main axis. To this end it is necessary to refer back to the previous generation. If the left-hand cell of that generation is differentiating into a branch cell, then the right-hand position of the undifferentiated cell at the new mode may be taken as assurance of its presumptive branch relations. Likewise position is the chief diagnostic criterion for the identification of undifferentiated branch zoöids.

The generalized pattern of branch development given below concerns only the position of the cells produced, for as will be seen later, the descendants of the median microzoöids are not always equivalent in their prospective values. The symbol X (or x) is used to denote branch generations in general (Fig. 3).

Lateral extension of the stalk bearing the median microzoöid X continues until the cell divides. Microzoöid X gives rise to two structurally similar cells, microzoöids $1X$ and $1x$. Microzoöid $1X$ occupies a median position with respect to the main axis of the colony, whereas $1x$ is the lateral member. Actually $1x$ immediately assumes the terminal position on the branch axis, thus constituting the growing point of the branch. Marked differential behavior now characterizes the two sisters: $1X$ lays down a short segment of stalk and then remains quiescent for some time; on the other hand, $1x$ actively extends the branch axis, then divides again within a few hours. Microzoöid $1x$ then produces $2x^1$ (median) and $2x^2$ (lateral). Cell $2x^2$ remains behind as a common nutritive branch zoöid while $2x^1$ assumes the terminal

position and continues as before, giving origin to $3x^1$ (lateral) and $3x^2$ (median); $3x^1$ to $4x^1$ and $4x^2$, etc. Each time the alternate secondary daughter is left behind as a common zoöid (Fig. 1).

Further mitotic activity in the common branch zoöids $2x^2$, $3x^2$, $4x^2$, etc. was not characteristic of the normal colonies whose histories were charted. About 2 per cent of the total number of colonies under surveillance showed a tendency toward precocious development. In these cases the repeated divisions of all branch zoöids led to such complex formations that the lineage soon became impossible to follow.

Conversely, microzoöid $1X$ on branches up to about level J commonly divides once, forming $1X^1$ (median) and $1X^2$ (lateral). Its mitotic activity on more distal branches is infrequent. On several

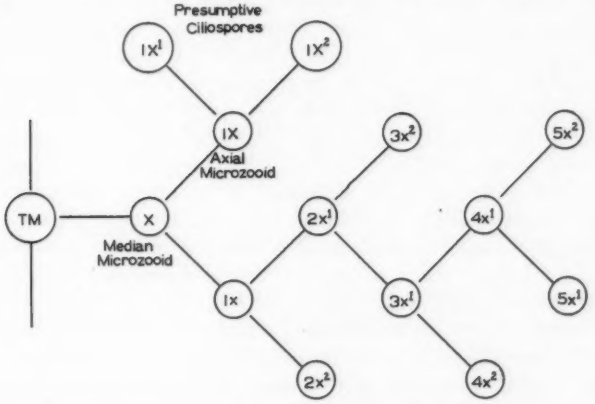

FIG. 3. Schematic representation of branch development.

branches of each colony the cell $1X$ or its descendants metamorphose into the migratory ciliospores which break away from the parent colony, leaving behind a short, stubby peduncle. Occasionally $1X$ produces a second or sometimes a third generation thereby augmenting the number of propagatory zoöids.

DIFFERENTIATION

The distribution of the heteromorphic zoöids to be described is a generalized account based upon a tabular evaluation of 200 cases of normal and experimentally cut colonies (proximal portions only), many of which were lost before complete development. Inclusion of the basal parts of operated colonies in the tabulations seems to be justified since statistical summaries of both normal and operated

colonies indicate no consistent variation as regards either rate of development or position of the differentiating zoöids.

During the life history of a colony six types of zoöids may be formed although not all of them are apt to be present at any one time.

1. The *common nutritive microzoöids* constitute the majority of the colony cells. They are the trumpet-shaped individuals characteristic of the genus. Notwithstanding the fact that most of them never assume any other form, they may be regarded as generalized components of prime colonies because (*a*) under undisturbed laboratory conditions some of them at random positions along the branch axis metamorphose into microgamonts, and (*b*) experiments may be performed in which variously situated common zoöids may be induced to differentiate into actively reproducing apical cells. Their capacity to differentiate into another type under imposed conditions gradually diminishes in time so that in older colonies the "vegetative" function becomes fixed. They can then be considered as somewhat specialized zoöids. The shape of zoöids that have persisted for relatively long periods of time without division are decidedly more slender and elongate than the "average" zoöids. This appears to be a modification of form rather than an alteration of volume. The extension of the protoplasmic body as well as its peduncle also appears to be gradual and continuous subsequent to the post-mitotic growth period.

2. Each colony bears a single *terminal macrozoöid* (*TM.*) at the apex of the principal axis (Figs. 1 and 2). In the earlier stages of colony formation three structural peculiarities clearly distinguish it from the common zoöids. It is considerably larger than any of the common types for the first ten or more axial generations, the body is characteristically flexed, and numerous annular striations in the pellicle are very pronounced. After ten generations or so its form individuality progressively diminishes. At levels beyond the twentieth branch only its position and generative activity are distinctive.

3. The *terminal branch zoöids* ($1x$, $2x^1$, $3x^1$, . . .) are almost diminutive replicas of the terminal macrozoöid. For the first few generations they are identified shortly after dividing by fairly marked pellicular annulations and a moderate anterior flexure of the tapering body. Near the close of branch development their patent features are obscure. Standard criteria of position and, to some extent, proclivity toward more rapid stalk formation and division must be relied upon to distinguish them from their common neighbors, the secondary daughters. Finally, at the terminus of a fully formed branch the last pair of zoöids are usually indistinguishable. This point represents about four generations on branch *A*, ten at intermediate levels (e.g. *H*), and progressively fewer after that (Fig. 1).

4. The most striking of the heteromorphic zoöids are the immature *ciliospores*, or asexual propagative cells which represent differentiated microzoöids of the order $1X$ or descendants. It is important for later discussion to note that the function of ciliospore formation is normally but not invariably restricted to these microzoöids. They have been observed to arise from microzoöids $5d^2$ and $6d^2$ in one instance, and $10h^1$ and $10h^2$ in another. Fauré-Fremiet (1930) found that the terminal macrozoöid was sometimes transformed into a migrating ciliospore but, unlike the usual ciliospore-forming cells, was unaccompanied by an endomictic reorganization.

Prior to ciliospore formation microzoöid $1X$ undergoes a profound growth in size. Very accentuated concentrically disposed pellicular furrows appear during the growth process. It is impossible to predict whether or not it will divide, for in a small percentage of the cases it is transformed directly into a motile ciliospore thereby terminating the lineage of that cell on the colony. Division of $1X$ in from 20 to 70 hours after derivation from the median microzoöid X is the rule, however. It is noteworthy, perhaps, that fission may occur after the onset of differentiation. The increased volume at this time is considerably in excess of the pre-divisional growth of the other zoöids. The two descendants, $1X^1$ (median) and $1X^2$ (lateral), are seldom equivalent in their size relations; the lateral daughter is usually the larger and the first to metamorphose.

Complete differentiation into motile cells already characterized involves the appearance of a girdle of long, closely set cilia about the equatorial region, the accentuation of the annulations, introversion of the oral disc, and differential growth in the plane of the diameter, eventually flattening the zoöids into disc-shaped bodies. It is believed that they are able to feed to some extent when the oral disc is introverted and the adoral zone folded. The gullet remains open and the undulating membrane within persists in its activity. The final metamorphosis of $1X^1$ may be deferred until a second generation of potential ciliospores ($1X^{11}$ and $1X^{12}$) are produced. The origin of at least four ciliospores from some axial microzoöids has been observed. Whether or not they represent three generations of alternate development or two generations formed dichotomously is still an open question.

The production of ciliospores is restricted to a limited number of loci on any given colony. Commonly there are but three or four such loci on one colony, although one protocol shows as many as seven. A generalized colony as visualized from tabulated data bears florescences at levels *D-K-P-T-AA*. They have been recorded for all nodes along

the axis between *B* to *W* inclusive, but never at consecutive nodes in any one case. It should also be stated that 1*X* on many branches divides without giving rise to ciliospores. Its descendants persist as common microzoöids.

5. *Microgamonts* (*microgonidia*, Wesenberg-Lund, 1925; *microconjugants*, Furssenko, 1929) develop from otherwise visibly undifferentiated microzoöids at the terminal or sub-terminal position along branch axes. The observed time interval between the formation of the microzoöids and their complete transformation into mature, liberated swimmers varies between the limits of six to thirty-six hours. The first sign of approaching metamorphosis is the appearance of a faint furrow about the equatorial region of the tapered body. The zoöid is thus marked into a larger peristomal portion and a smaller basal portion. Cilia gradually grow out from the body at the furrow. At first very short, they soon develop into a broad girdle whose movements simulate those of a slowly waving undulating membrane (Fig. 4). Meanwhile the diameter of the cell increases. The oral half

FIG. 4. Differentiating microgamont about 5 hours before the completion of metamorphosis. × 250.

containing the retracted peristomal disc is transformed into the slightly flat, sub-conical oral end of the swimmer. The posterior portion is further reduced to a very slightly convex aboral surface at whose center the stalk is still attached. Its struggling movements gradually accelerate until at full maturity it breaks away from the parent colony. At full term the microgamonts resemble ciliospores in their eccentric body outline, ciliature, and general movements. They are, however, not perceptibly larger than the common zoöids, show but faint indications of annulations, and swim more rapidly.

While cytological studies were not attempted, it is definitely established that these are the migratory members of the copulatory series. When liberated some have been observed to swim rapidly back and forth in an arc about the parent colony, occasionally coming to rest on some zoöid, or crawling about on the branches and over the individuals in various positions, and eventually swimming away as though its relatives were not receptive of its attentions. The duration of the migratory phase was not determined. One was observed to settle upon terminal macrozoöid No. 3 of a young colony possessing nine cells in all. Two and one-half hours later the aboral end of the

migrant was firmly fused with the lateral surface of the apical cell. By that time the ciliary crown of the swimmer had been resorbed.

As regards origin, it is frequently difficult to determine if microgamonts arise from prospective terminal or sub-terminal branch microzoöids because of the facility with which the prospective values of very recently produced daughter cells may be altered. For example, if the terminal cell (e.g. $2x^1$) is accidentally lost, the sub-terminal cell ($2x^2$) immediately assumes the terminal functions. If loss occurs soon after fission, when both daughters are superficially alike, the stalk remnant left behind at the node occurs in the alternate position, whereas the cell which normally would have been the alternate ($2x^2$) becomes the growing point. The tendency of the terminal branch cell to differentiate into a microgamont is conclusively demonstrated by the successive metamorphosis of sister cells. This means that the continued lateral growth of a branch subsequent to the production of a microgamont may represent each time an early (undetected) transformation of the secondary zoöid into a new terminal cell. The confusion is apt to arise because the alternate position of the stubby stalk left by the lost cell or by the migrating microgamont cannot always be diagnosed accurately. Fortunately in many instances the terminal branch cell is well indicated either by position or by symptoms of an approaching division by the time the alternate cell shows signs of becoming a microgamont.

Tabulations show that 85 per cent of the microgamonts observed on colonies whose complete developments were charted, were produced by the four basal branches *A*, *B*, *C*, and *D*, enumerated in decreasing order of importance. The rest arose from zoöids on branches *E*, *F*, *G*, and *K*. They were formed either by the terminal ($-x^1$) or alternate ($-x^2$) cells as far out on the branches as the sixth generation on *A*, *B*, and *C* (including $5a^{21}$ and $5a^{22}$), the seventh generation on *D*, and the eighth generation on *F*. None were observed to come from the median microzoöid *X*, the axial microzoöid $1X$ or its descendants.

The majority of the colonies bore but one or two microgamonts; a few produced as many as nine but not more than three for any one branch. They occurred simultaneously on different branches but successively from the alternate zoöids on any given branch. And finally, it is important that they differentiated only from branch zoöids at or near the terminal position, never from the more axial (older) microzoöids.

6. *Macrogamonts* were found only in the terminal macrozoöid position on the primary axis. Attempts to discover some morphological or behavioral distinction between terminal macrozoöids and pre-

conjugant macrogamonts were negative, but this possibility is not precluded since of the three or four hundred colonies inspected, none may have happened to have been preparing for conjugation. The presence of an attached microgamont constituted the sole criterion for identification.

Conjugants at all levels between the terminal macrozoöid positions three to twenty-four inclusive were observed. The youngest colony possessed but nine cells in all, including the conjugant. Two successive conjugants, five generations apart, were recorded for one colony.

The incidence of conjugation reached a maximum during the month of July; before or after that the conjugants were quite rare. Even in July only about 5 per cent of the colonies bore conjugants although more of them were producing microgamonts. Many of the colonies produced no gamonts whatever during the period of development on the slides.

Some of the effects of conjugation upon colonial development will be treated in a later section.

Reactive Capacities

The motor responses of *Zoöthamnium alternans* are developed to a relatively high degree. Very local reactions by individual zoöids result in the retraction of the ciliated oral disc. More general responses evoked by altered salinity of the water, sudden contact stimuli, etc., are expressed by an extremely rapid contraction of the stalk and the zoöids. Gradual relaxation or extension ensues within fifteen to thirty seconds unless the stimulus is sustained. This manifestation of irritability constitutes one of the major technical difficulties for experimental work. When the colony is contracted, it is practically impossible to distinguish a given zoöid or to cut the stalk at a desired point. Fortunately for the experimental work the stalk alone may be touched gently without disturbing the colony. A series of attempts to inactivate the colonies by subjecting them to a variety of narcotics in various concentrations were quite unsuccessful. Freezing temperatures did not modify the contractions to any great extent.

Summary

1. The frond-like colonies of *Zoöthamnium alternans* develop according to a well-determined pattern. This development is described with particular reference to the origin of the heteromorphic zoöids.

2. An asexual propagative zoöid (ciliospore) detaches from a mother colony, swims away, and settles down to form a new colony. When affixed to the substrate the ciliospore begins to secrete a peduncle or

stalk. The first portion of the peduncle is flexible but not contractile. From a point some 200 to 300μ above the hold-fast, the ciliospore suddenly begins to produce a contractile cord in the core of the hyaline stalk substance. Distal to this point the contractile cord is an integral part of the stalk; it is continuous from branch to branch and from cell to cell.

3. When the stalk is approximately 500μ long the ciliospore divides unequally. The larger daughter remains axial in position. It represents the first generation of the terminal macrozoöid series (*TM.* No. 1). The smaller lateral cell is the initial zoöid of the first branch, the median microzoöid *A*.

4. Successive divisions of the terminal macrozoöid produce each time a terminal macrozoöid of the next generation and a median microzoöid. The latter are strictly alternate in position: they alternate on right and left sides of the primary axis at successive nodes. As many as thirty-three generations of the terminal macrozoöid have been observed.

5. The first division of the initial branch zoöid gives rise to an axial microzoöid and a lateral stem cell. The stem cell generates alternating zoöids of the main branch strain whereas the axial microzoöids on some of the branches represent the presumptive ciliospores.

6. At least four types of zoöids comprise a colony: (1) a single terminal macrozoöid at the apex of the primary axis, (2) many common microzoöids scattered along each branch axis, (3) a terminal zoöid at the tip of each branch, and (4) immature ciliospores which arise from axial microzoöids on some of the branches. During epidemics of conjugation two more types may be formed: the terminal macrozoöid may be transformed into a macrogamont, and common microzoöids at random positions may metamorphose into migratory microgamonts. The spatial distribution of the heteromorphic zoöids is described in some detail.

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FORM REGULATION IN ZOÖTHAMNIUM ALTERNANS

F. M. SUMMERS

(From Bard College, Columbia University)

Introduction

Interest in morphogenetic studies has long centered about the part-whole relationships obtaining throughout the formative period in organic development. In so far as a system comprising several parts is concerned, the term *organization* implies the existence of integrating factors that condition to some extent the limits and direction of regional specialization. A remarkable number of investigations on metazoan organizers have already demonstrated the importance of extrinsic factors upon determination in specific parts. It was felt that additional information about these factors could be gained by applying operative techniques to an animal type in which, presumably, the interrelationships have not attained so great a degree of complexity.

The principal endeavor of this work was to investigate some of the qualitative and quantitative aspects of growth and differentiation in *Zoöthamnium* colonies, regulating after the removal of actively growing (distal) parts, and to compare regulative behavior with the normal development already described by Fauré-Fremiet (1930) and Summers (1938). The results have made it possible to offer a rough map of potencies and prospective values of individual cells at various positions in the colonial pattern and to indicate some of the changes in the expression of inherent potencies which may be induced by experimental means.

It is a pleasure to thank Professor L. R. Cleveland of Harvard University for extending to the author the privileges of his laboratory during January, 1937.

Materials and Methods

The materials and techniques used in this study are similar to those described in the previous paper and need not be repeated here. The only additional detail pertains to the method of shearing the stalk. Two fine scalpels made from No. 9 sewing needles were used for the purpose. One was brought to rest against the surface of the stalk and the other sheared against it in scissors fashion.

The plan of attack is by no means new, but the type of organization

dealt with seems to promise a fresh approach to the current problems of form determination. A colony of *Zoöthamnium alternans* is admirably adapted to work of this kind by virtue of the regularity and precision with which the characteristic colonial pattern develops. The alternating arrangement of the branches and cells makes it comparatively easy to follow the history of any one cell throughout the course of its development for evidences of growth, division, or differentiation. The spatial relationship of the cells minimizes to a great extent some of the factors so difficult to evaluate for compact tissues. Crowding effects such as mutual contact, pressure, etc. (Peebles, 1931) are of no great concern here. Then, too, the separated cells are uniformly bathed by an almost constant medium, filtered sea water. Physiological relations between them are effected through a well-defined channel, the stalk with its neuro-muscular cord (Fauré-Fremiet's "cordon central"), rather than through the general expanse of juxtaposed cell membranes.

Regulative Development

Standards of Judgment.—Colonies maintained for several days on slides are apt to be attacked by internal parasites or covered by plant growths of one kind or another, especially in the basal regions. When operations are made the axial growth of a colony is retarded for an average of 23.1 hours pending the formation of a new terminal macrozoöid. It is during this period of arrested development that adverse environmental conditions are liable to bring about an incapacitation or loss of important zoöids before a decision relative to the success of the operation can be reached. A small proportion of the successful operations shown in Tables I and II do not appear in subsequent tables because they were destroyed or abandoned after indubitable signs of new terminal macrozoöid differentiation had appeared but before descendants were produced. In the absence of a regenerate, the responses were recorded only when all of the structural characteristics of the new terminal macrozoöid were established and, in addition, the "activated" branch developed an anterior flexure. In consequence of the stalk curvature the new terminal macrozoöid assumes the apical position upon an anteriorly directed axial stalk. The point of curvature marks the node (Figs. 3 and 5) at which the stalk suddenly increases to a diameter approaching that of the original axis.

Regenerative responses were arbitrarily called negative only when one of the following conditions were realized: (*a*) there was no activity for at least 48 hours; (*b*) in the event that mitotic activity continued for a generation or two, a minimum of 72 hours was allowed for signs

of regulatory activity; (*c*) when the terminal branch zoöid in the line of succession metamorphosed into a migrating zoöid of some kind. The thirty-seven negative cases shown in Tables I and II were maintained for a mean time of 94.3 hours after the last division, with extremes of 52 to 212 hours.

TABLE I

		PARTS CUT AWAY								
		TM+0	TM+1	TM+2	TM+3	TM+4	TM+5	TM+6	TM+7	Total
LEVEL OF OPERATION	Q	1								1
	P	(1)	(2)		(1)					(4)
	O	2 (1)	1 (1)	(1)	(1)					3 (4)
	N		(1)							(1)
	M	1 (1)	(1)							1 (2)
	L	(1)	(1)							(2)
	K	1		(1)	(1)					1 (2)
	J	2 (1)								2 (1)
	I		1	1	1	(1)				3 (1)
	H	1	(1)				(1)			1 (2)
	G	3 (1)	1 (1)	(1)	(1)					4 (4)
	F	4	1							5
	E	1	1		1					3
	D	3	2							5
	C	6	4		1	1				12
	B	3 (1)	4 (1)	2		1		(1)		10 (3)
	A	9 (6)	6 (3)	6	2 (2)	1	1	1	1	27 (11)
Total		37(13)	21 (12)	9 (3)	5 (6)	3 (1)	1 (1)	1 (1)	1	78(37)

The distribution of regulative responses in 115 operated colonies summarized according to the number of apical branches cut away: *T.M.* + 0 = only the terminal macrozoöid removed; *T.M.* + 1, *T.M.* + 2, etc. = terminal macrozoöid plus one, two, or more branches removed. The letters in the left-hand column designate the branches from which the regenerates arose. The numbers of cases in which no response occurred are shown in parentheses. The first responses of the successively operated colonies are included. No attempt has been made to indicate the number of zoöids on the branches dissected away; it may be estimated by referring to Fig. 1 in the previous paper.

All colonies were abandoned when the important zoöids appeared to be unhealthy.

Adjustment of Descriptive Notation.—An arbitrary departure from the standard notation (see Fauré-Fremiet, 1930; or Summers, 1938) seems to be advisable in view of the complications arising from the

designation of generations produced by the secondary zoöid near the point of origin of the new terminal macrozoöid. To illustrate: if the new terminal macrozoöid differentiates directly from $2a^1$, then the secondary zoöid $2a^2$ continues to generate zoöids of the branch A, viz. $2a^{21}$, $2a^{22}$; $2a^{211}$, $2a^{212}$, etc., which complicates a system already difficult to summarize briefly.

At this point it is proposed to adjust the terminology so that the new terminal macrozoöid (actually $2a^1$) corresponds in position to the

TABLE II

		ORIGIN OF NEW TERMINAL MACROZOOID										
		IX	IX'	Ix	2x'	2x²	3x'	4x'	4x²	5x'	6x'	Total
LEVEL OF OPERATION	Q			1								1
	P			(1)	(1)		(1)			(1)		(4)
	O	2			1 (1)		(2)	(1)				3 (4)
	N						(1)					(1)
	M			(1)	1		(1)					1 (2)
	L				(1)					(1)		(2)
	K			1				(1)			(1)	1 (2)
	J			2	(1)							2 (1)
	I	1	1			1			(1)			3 (1)
	H						1		(1)	(1)		1 (2)
	G		1	2	1 (2)	(1)				(1)		4 (4)
	F			2	2	1						5
	E	1			1			1				3
	D			1	3			1				5
	C			8	3		1					12
	B			5 (2)	2	1	1	1	(1)			10 (3)
	A	1 (1)	(1)	15 (7)	5	(1)	5 (1)	1				27 (11)
Total		5 (1)	2 (1)	37 (11)	19 (6)	3 (2)	8 (6)	4 (2)	(3)	(4)	(1)	78 (37)

Lateral distribution of operations. This table summarizes the data from Table I according to regulation by branch generations irrespective of the amount of colony cut away. The left-hand column represents the branches from which the regenerates developed; headings indicate the position and generation of the zoöids which produced the new terminal macrozoöid. The negative responses are shown in parentheses.

original terminal macrozoöid (*T. M.* No. 1), but is distinguished from it by a prime (′) symbol. The lateral branch zoöid $2a^2$ becomes A', the first zoöid of a new branch at the original A level (Fig. 1).

This adjustment is valid if subsequent products are to be treated descriptively as new primary branches rather than as branches of the second order on the first branch below the cut. This is nothing more than a manipulation of terms to facilitate comparison of normal and regulating colonies.

Trauma.—For a number of reasons it appears probable that traumatic shock effects are not significant factors in post-operative adjustments of colonial form. Cells adjacent to the cut areas and elsewhere soon expand and feed as before. Processes of mitosis or

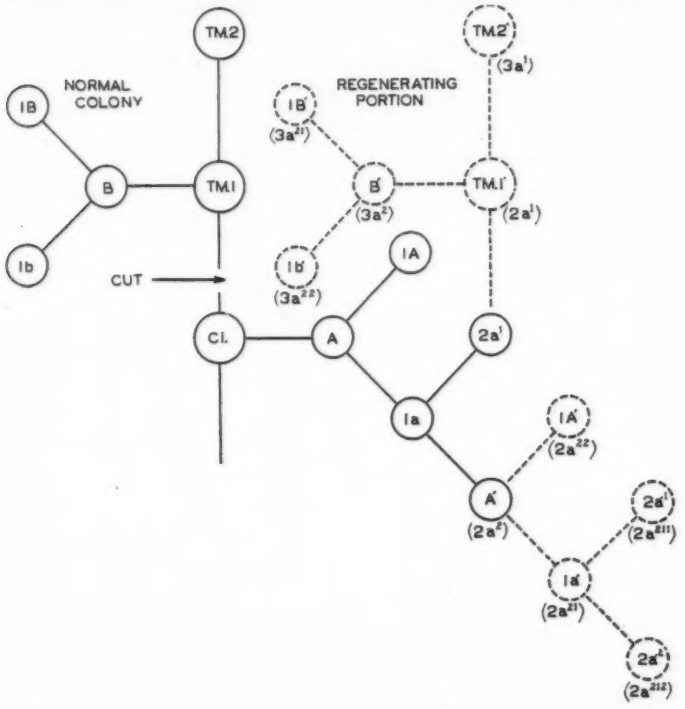

FIG. 1. Diagram of regulative development. The revised notation for designating cell lineage in regulating portions is indicated within the circles. The symbols in parentheses under the circles illustrate how cumbersome the conventional terminology would soon become if applied to the regenerates. The diagram shows the lineage of a regenerate from branch *A*. If the principal axis is severed between branches *A* and *B* at a time when there are three cells on branch *A*, the regenerate usually arises from the terminal branch zoöid $2a^1$ as illustrated. The sub-terminal zoöid ($2a^2$) assumes the terminal branch position *A'* and continues to generate branch cells. The new branch *B'* is produced by the first division of the new terminal macrozoöid *T.M.* 1'. The parts produced after cutting are drawn with broken lines.

differentiation, in progress at the time of operation, continue without perceptible interruption. Or these processes may begin at varying intervals after cutting, in any of the branch zoöids, except that which becomes the new terminal macrozoöid. Furthermore, when there is

no regulation, or when the median microzoöid divides one or more times before the new terminal macrozoöid is recognizable, the first branch below the cut continues to generate common zoöids as before. Relative to division rates, the available data indicate that, exclusive of the one which bears the presumptive terminal macrozoöid, branch growth is not perceptibly altered after cutting. As a rule there is a lag in the development of the activated branch pending the differentiation of a new growing point.

The only effects of mechanical disturbance are evidenced for a very short time after cutting by a state of irritability during which the contraction of decapitated colonies is frequent, irregular, and sometimes tetanic. But normal overt behavior is resumed within a few moments when the stalks are shorn cleanly at internodal points. Operations were considered acceptable only when the normal reactions were regained within a relatively short time. Cases where only the neuro-muscular cord of the stalk was injured are to be treated in another section.

Distribution of Operations.—A general résumé of the experimental results in terms of initial regulative responses in *Zoöthamnium alternans* is given in Tables I and II. Of the 144 protocols at hand (acceptable operations), 78 yielded positive responses and 37 were negative; the remainder were inconclusive according to the standards chosen and are omitted in the digests.

Table I summarizes the responses to various types of cuts made at the several levels along the principal axis irrespective of the number of generations on the regulating branches. In Table II the same protocols are tabulated according to regulative responses by the various branch generations without regard to the number of branches or zoöids removed. In this table the negative cases show only the zoöids which were expected to reconstitute the axial growing point; some of these, failing to regulate, continued to develop laterally without further differentiation.

The symbols X and x are used to indicate generations on a generalized branch (Summers, 1938). With reference to a specific branch, e.g. branch D, the symbol $1x$ refers to the microzoöid $1d$, and $1X$ means axial microzoöid $1D$.

Simple Cut-offs.—In general when the terminal macrozoöid was cut off, the terminal cell of the first branch below the cut differentiated into a new, well-defined terminal macrozoöid whose first and subsequent divisions proliferated the alternating median microzoöids (initial branch cells) of the regenerate. Some operations were made at a time when a single cell, the median microzoöid X, represented the

adjacent rudimentary branch. In all such instances recorded at least one division followed without perceptible delay, thus producing an axial microzoöid $1X$ and the presumptive terminal macrozoöid $1x$. More frequently the removal of a terminal macrozoöid left two or three cells on the last branch (Fig. 1). Simple cut-offs leaving this branch with more than three cells were rarely possible for the reason that a division of the terminal macrozoöid usually preceded the third division on the adjacent branch.

There were a number of cases where, after an operation, the appointed branch continued to develop at a normal rate for one or more

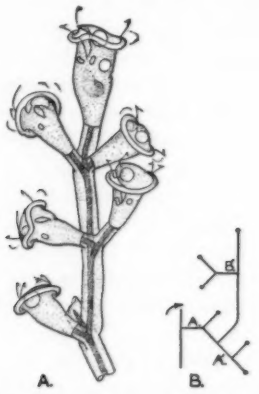

FIG. 2. *A*. Colony *3H3-5* drawn approximately 53 hours after the operation. The apical cell was destroyed at the two-cell stage of development. The remaining microzoöid *A* divided twice before the new *T.M.* differentiated from $2a^1$. Its first division produced the initial cell of the branch *B'*. The alternate daughter ($2a^2$) extended the original branch, and the axial microzoöid $1A$ persisted without further change. × 250.

B. Schematic representation of the resulting growth.

generations before another terminal macrozoöid differentiated (Fig. 2). In approximately 12 per cent of the regulating colonies two divisions followed the operation, within normal time limits, before indications of a new terminal macrozoöid appeared. In one case there were three pre-differentiational divisions.

Regulation from the terminal branch zoöid $1x$ following simple decapitation of the growing point occurred in a high percentage of the cases (see Tables I and II). As far as the various levels along the primary axis were explored, the cells of the first few branch generations exhibited relatively frequent regulative responses. For the small number of operations made at high levels on old colonies, the transfor-

mation of $1x$ into a new terminal macrozoöid occurred with about the same frequency and with as much dispatch as for earlier periods, i.e. lower levels on younger colonies. The metamorphosis of the activated microzoöid $1x$ at levels above *E* required no more than the average time necessary for differentiation of apical cells produced at lower levels. This appears to correlate with the more or less uniform rate of normal axial growth (Fauré-Fremiet, 1930). Likewise $2x^1$ zoöids on the various branches responded in the majority of trials. Zoöids of the $3x^1$ generation or later failed to regenerate above the mid-region of the experimental colonies.

In order to test the responses of a branch cell of the third generation it was necessary to cut away the newer branches which had formed above it along the main axis. In tests of the fourth or later branch generations, a relatively large part of the colony had to be removed.

Compound Cuts.—When the terminal macrozoöid *and* the terminal cell of the last branch were removed, the terminal cell of the second preceding branch was frequently induced to differentiate into a new terminal macrozoöid. This particular relationship obtained for a limited number of successive branches and even then was unpredictable. For instance, the terminal macrozoöid was sometimes produced by the sub-terminal (a secondary) microzoöid of the newest branch despite the presence of a healthy terminal cell on the penultimate branch. In rare cases the latter assumed the regulative function in the presence of a complete uninjured branch between it and the cut-off.

The number of regenerates obtained from the sub-terminal branch zoöids is given in Table II. Microzoöids of the order $2x^2$ responded up to the level of branch *I*, whereas sub-terminal zoöids of the fourth generation ($4x^2$) did not respond at all. It is also certain that some colonies did not regenerate from either the sub-terminal zoöid of the first branch below the cut or from the terminal zoöid of the next adjacent branch. These branches continued to develop in a normal fashion for one or more generations without attempting to produce a new terminal macrozoöid.

The reactions of the older segments of well-developed colonies were tested by means of extensive "cut-backs," colonies cut off at some more basal internode. The data obtained (Table II) suggest an inverse relation between the number of regulative responses and the age of the activated zoöids, i.e. the frequency of responses diminishes as the number of lateral generations increases. The frequency of negative cases even in younger generations increased in the high levels, which is probably an expression of the fewer generations required to bring the more distal branches to full development. The

same data arranged according to amount of colony removed (Table I) show that basal branches may differentiate a new terminal macrozoöid after as many as seven branches plus the apical zoöid are cut away.

The cut-back experiments were successful only as regards the demonstration of initial reactivity to surgical alterations. The cases in Tables I and II where relatively large portions of the colonies were removed show only that the prospective values of certain zoöids along branch axes may or may not be modified, depending upon the amount of colony dissected away. The capacity of the responding zoöids for sustained growth is not known because all of the colonies cut back four or more branches had to be discarded before the regenerate attained full growth. Indeed, some were maintained under experimental conditions only long enough to produce a new apical cell. The chief difficulty is referable to the fact that the basal branches were the first to be attacked by vegetable growths propagating over the surface of the slide. The affected basal zoöids were shed before the colonies reached maturity. For this reason the zoöids of branch *A* on colonies with eight or more branches were usually unsuitable for testing. The age of the colony at the time of operation plus the additional time required for the differentiation of one of its zoöids gave to the parasites an advantage that was too frequently fatal to the experiment.

Regulation from the Axial Microzoöid Series.—Although nearly 9 per cent of the regenerates sprang from the axial microzoöid (1*X*) series, their regulative behavior was capricious and could not be induced at will. One of the most striking facts in this connection was the origin of new growths from 1*X* or descendants on complete, uninjured branches (Fig. 3). One originated from the third branch below the cut. Deliberate attempts to activate a given axial zoöid by eliminating all other zoöids on the branch resulted in (*a*) no further developmental activity, or (*b*) regeneration from some zoöid on the next lower branch. A three-cell colony which was trimmed down to a single cell, the axial microzoöid 1*A*, remained without further change for 165.5 hours; its contractile and feeding responses appeared to be normal for the entire period of observation. Similarly, the corresponding zoöids on branches *E*, *L*, *M*, and *P* of other colonies were tested without avail. In each case the regenerate developed from zoöids on the next lower branch and is so recorded. On the other hand, it has been demonstrated that the axial microzoöids are capable of regenerating (see 1*X* and 1*X*[1] in Table II).

The protocols show that 1*X* or some of its descendants carry terminal macrozoöid potencies. In some of these cases the regenerates

formed directly from $1X$ without leaving ciliospore-producing cells on the activated branch. The others regulated after $1X^1$ and $1X^2$ were formed; $1X^1$ gave rise to the new terminal macrozooïd while $1X^2$ produced one or, after division, two typical ciliospores.

Regulation after Successive Cuts.—The successive operations were of two general classes: (*a*) progressive, in which the second and third

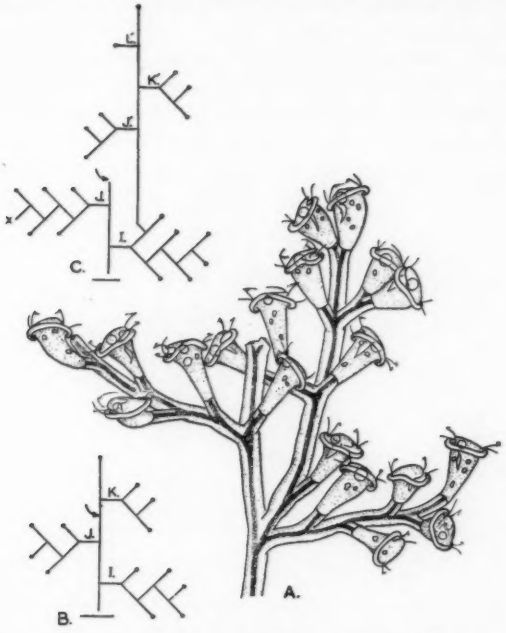

FIG. 3. *A*. Drawing of colony *3IJ-2* approximately 72 hours after cutting. In this case the regenerate was produced by the axial microzooïd on the intact branch *I*. Axial microzooïd $1I$ divided before the new terminal macrozooïd differentiated: $1I^1$ regulated, leaving $1I^2$ near the base of the new axis. An increase in diameter of the new axis is evident near its junction with the original colony axis. × 250.

B. Condition of the apical end of the colony before cutting at the point indicated by the arrow.

C. Schematic representation of the resulting growth at the time the drawing was made.

cuts removed regenerated parts distal to the preceding operation (Fig. 4); and (*b*) regressive, where the entire regenerate plus additional parts of the original colony were cut off. In the first group the second and third regenerates were themselves products of regenerated segments. Those of the second group developed from some more basal

cell of the original colony. In this respect they were similar to the extensive cut-back types and subject to the same technical limitations.

The regulative activity of the zoöids of the second and third order regenerates in progressively cut colonies was similar in most respects to that evoked after simple cut-offs. In each of the nine cases studied the same morphological pattern and, as far as the limited number of cases permit judgment, similar developmental rates obtained after the second and third operations.

Incomplete Section of the Stalk.—In a few cases out of many trials a local injury to the neuro-muscular cord was effected without destroying the continuity of the cortical hyaline stalk substance. A requisite degree of compression between the needles caused the cord to break down into a series of irregular protoplasmic droplets, some of which appeared to be independent of any attenuated membranous connectives.

FIG. 4. *A*. Development after two successive operations (54 hours after the first cut). The original axis lies on the right. The severed peduncle of the regenerated terminal macrozoöid may be seen near the base of the second regenerate. Axial microzoöid 1*C* was badly parasitized; it dropped away soon after the drawing was made. × 250.

B. Schematic representation of *A*. The cuts are indicated by arrows. *Parasitized zoöid.

Several interesting facts were brought out by this type of operation. The severed part of the neuro-muscular cord did not recover from the injury, i.e. the structural or functional continuity between the separated parts was not re-established. After the injury there was no subsequent degeneration of the cord in either proximal or distal parts. The functional unity of the whole was permanently impaired. The

proximal and distal parts contracted independently of each other and, as established in one case, the distal portion continued to grow and differentiate in the manner of an intact colony, whereas the proximal part regenerated a new primary axis from a zoöid below the injury (Fig. 5).

More substantial data are obviously required before definite conclusions can be drawn. Nevertheless these results do suggest interesting possibilities for further study. A thoroughgoing investigation of the contractile, transportative, and transmissive properties of the neuro-muscular cord may lead to a further elucidation of coördinating factors in colony formation. At least here is a clear indication that, whatever the physico-chemical nature of the integrative factors, they are probably mediated through the substance of the cord.

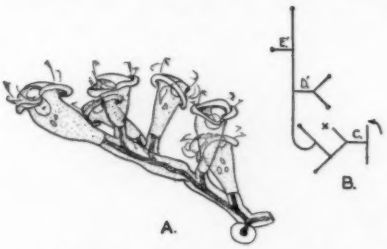

FIG. 5. *A*. Branch *C* of colony 9*B*4 56 hours after injury to the neuro-muscular cord (drawn from above). The original colony of six branches was pinched in the mid-region, isolating *ABC* from *DEF* plus the terminal macrozoöid. The terminal cell on branch *C* ($3c^1$) at the time of the operation differentiated into a new terminal macrozoöid which produced two new branches as shown. Note the increased diameter of the stalk just lateral to the second zoöid. This marks the position of the microzoöid $3c^1$ at the time of injury. × 250.

B. Schematic representation of branch *C* as drawn. Axial microzoöid 1*C* was accidentally cut away from the position marked (*x*).

Several completely isolated fragments were followed for a time by transferring every few hours to fresh filtered sea water. Nothing of unusual interest occurred in their development. Growth, differentiation, and regulation in progress at the time of cutting continued as before for the few generations that were followed. They did not re-attach to the substrate but developed as free-swimming fragments. Their growth capacities or the minimum size necessary for survival were not investigated.

Differentiation in Regenerated Parts.—The regenerates formed on decapitated colonies, after single or successive operations, are capable of producing any of the six types of heteromorphic zoöids previously described (Summers, 1938). According to the data compiled from

77 protocols the type, number, and distribution of zoöids on the regenerated parts compare favorably with the control colonies. The regenerates consisted of a new terminal macrozoöid, varying numbers of common microzoöids, a terminal cell at the tip of each branch, and one or more potential ciliospores, depending upon the degree of development following an operation. Macro- and microgamonts likewise differentiated on regulating parts with about the same frequency and vertical distribution as for corresponding regions of normal gamont-producing colonies. Regenerates sometimes differed from the controls in respect to the branch generation involved in the production of ciliospores and microgamonts. In normal colonies the ciliospores developed not earlier than the $1X$ generation and the microgamonts only from $2x^1$ or succeeding generations. On the regenerates one or the other of these two types of migrating zoöids frequently developed from the initial branch zoöid (Fig. 6*B*). This tendency towards

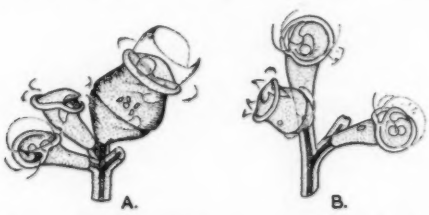

FIG. 6. *A*. Terminal macrozoöid 13′ (above branch *M*) differentiating into a ciliospore 204 hours after cutting. The colony was sectioned between branches *E* and *F*. × 250.

B. Metamorphosis of the median microzoöid *S*′ into a microgamont. The drawing was made 127 hours after regulation from the axial microzoöid 1*O*. × 250.

earlier differentiation obtained not only in the young regenerates on immature colonies but also in those derived from older (basal) zoöids of nearly mature colonies.

Another noticeable deviation from the established norm was the occasional metamorphosis of the terminal macrozoöid into a migratory zoöid, either ciliospore or microgamont (Fig. 6*A*), thus bringing to a close the growth along that particular axis. The terminal macrozoöid of the regenerate may differentiate directly into any of the three reproductive zoöids: microgamont, macrogamont, or ciliospore. Fauré-Fremiet (1930) reported the formation of the latter type in rare instances during the normal development of this species but the process was unaccompanied by the endomictic process which he described for normal ciliospore development.

While studying conjugation in *Zoöthamnium arbuscula*, Furssenko

(1929) observed that a local injury to one of the main branches affected the zoöids distal to the region of injury, inducing many of the microzoöids to metamorphose into microgamonts ("microconjugants"). Similar effects on the whole colony were induced by unfavorable environmental conditions, e.g. inanition or lack of oxygen. He did not observe a regulatory response subsequent to the injury.

Regulation after Conjugation.—From the following fragmentary account of the growth activity manifest in conjugating colonies it is at once clear that this aspect of development alone constitutes a lengthy research problem. Only ten of the colonies whose lineage was being followed happened to conjugate so that a detailed analysis

FIG. 7. A colony several days after the onset of conjugation at the level of branch *N*. The ex-conjugant divided into a cluster of large zoöids, one of which differentiated into a new *T.M.* whose further development prolonged the main axis. The exact lineage of these cells is not known. × 250.

of the process is not immediately available. Much of that which follows is based upon conjugants observed among the adventitious growths on the culture slides whose histories are but imperfectly known. Conjugation is introduced here because it affords one clue to qualitatively different physiological relations between the apical zoöid, the conjugant in this case, and a large area of the subordinate regions of the colony.

So far the results obtained from regulation experiments bespeak a regular functional correlation between the single cell in the apical position and the zoöids in sub-adjacent regions, such that the latter

are subservient to the former. Potentialities known to be present in zoöids of a lower order are presumably held in abeyance by the apical influence. Cutting away the apical region evokes a response in some zoöid in a subordinate but adjacent region. The response is a differentiation of another apical zoöid whose relations with the whole are seemingly homologous with those of the original apical cell. There may be a time between decapitation and subsequent regulation when the apical cell influences are altogether absent, yet the interim is not sufficiently great to seriously modify the observable growth phenomena. It is noteworthy that subordinate branches attain about the same end-point of lateral growth in control and decapitated colonies.

As stated in the previous work (Summers, 1938), the macrogamonts were observed only in the terminal macrozoöid positions 3 to 24 along the primary axis. The fusion of gamonts invariably brought axial development temporarily to a close some 12 to 13 hours after the last mitosis.

The conjugant remained quiescent for periods of about four days, then divided into two moderately large zoöids. One of the two ex-conjugants assumed the form of a terminal macrozoöid and resumed axial development after the four-day interruption. The fate of the sister ex-conjugant is a matter for conjecture at present; some disappeared from the colonies between observational periods while others divided into clusters of from two to seven large ciliospore-like zoöids at the base of the new axis (Fig. 7). The histories of these are likewise unknown. Apparently they do not propagate additional axes while associated with the parent colony. The development of ciliospores from some of the ex-conjugants in *Zoöthamnium arbuscula* (Furssenko, 1929) is suggestive, however.

The point to be made relates primarily to the behavior of the colony as a whole following the conjugation process. Prior to the completion of conjugation and continuing thereafter a new growth phenomenon appeared. The first three or four branches below the presiding conjugant developed out of all proportion to the average expectations (Fig. 8). The number of branch generations was in some instances greater than twice that of corresponding branches in controls. Moreover, many of the common lateral or secondary zoöids were activated to divide one or more times, originating second order branches which, in turn, sometimes produced tertiary branches. In this way each of the first few branches below the conjugant level grew almost as individual colonies. The greatest lateral growth effect obtained nearest the conjugant and diminished basally as a gradient. The normal tendency toward a pyramidal colony pattern was thereby

reversed in the environs of the conjugant. More precise information regarding growth intensity and capacity factors in both the ex-conjugant strain and the subordinate branches awaits further investigation.

Discussion

One of the most important consequences of the work is the demonstration of qualitatively different physiological relations between

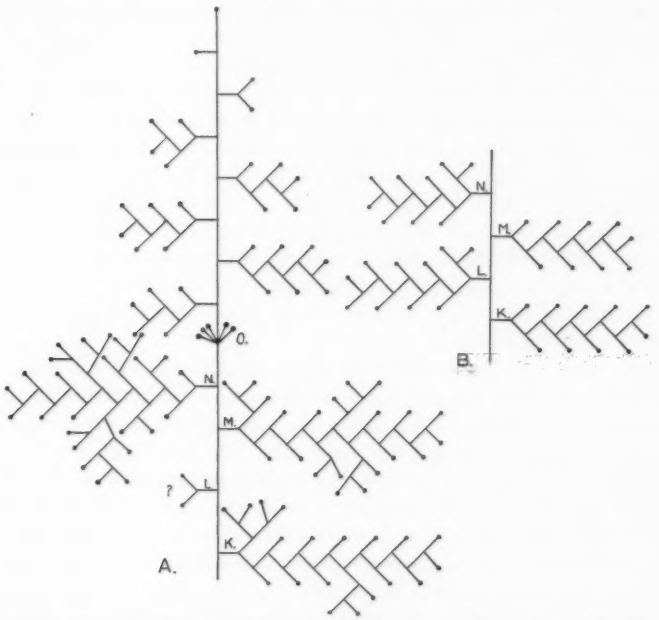

Fig. 8. *A*. Schematic representation of protocol 19*oe* to show the disproportionate development of branches *K*, *M*, and *N*. The lineage of branch *L* could not be deciphered. One of the ex-conjugant zoöids at *O* produced the apical growth illustrated in the diagram.

B. The corresponding portion of the largest of the 70 control colonies.

spatially separated cells. Under normal conditions a specific pattern unfolds. When an apical region of a colony is cut away some zoöid of a lower order, one whose complete developmental possibilities are otherwise never expressed, assumes the dominant generative functions, and the characteristic pattern perseveres. So far these results are intelligible in terms of what Child (1929) calls physiological correlation: the relations of dominance or control and subordination between

parts. He concludes "that dominance and subordination depend primarily on quantitative, rather than specific differences in physiological condition and that they represent a certain aspect of a physiological gradient." In *Zoöthamnium alternans* the transformation of a terminal macrozoöid into an ex-conjugant initiates an entirely different developmental phase which gives another clue to the general nature of apical control. Four days after the fusion of gamonts the normal growth relations of a varying number of branches below the conjugant level are upset in a rather remarkable way. Each of the three or four adjacent branches develops out of all proportion to the normal expectations. The precocious mitotic activity produces secondary and even tertiary axes on the affected parts. This unusual phenomenon does not occur when the terminal macrozoöid is present *or* when it is absent; it is effected by some new quality in the coördinating mechanism arising in consequence of conjugation activities in one particular cell—the apical cell.

The effects obtained after decapitation and conjugation certainly suggest that the single cell in the apical position is responsible in a large measure for quantitative and qualitative regulation of the part-whole relationship and that the control varies with respect to the local activities of the parts.

There also seems to be a coördination between the cells on different branches. A new terminal macrozoöid arising from one of the branch cells exerts its influence from what was formerly considered to be a branch position. Perhaps branch-to-branch coördination also explains the stable activity of the variously placed cells in the interim before a new apical cell differentiates; only one of the several possible zoöids regulates. The control of a terminal branch cell over the cells on its own branch can be interpreted in a similar way. As long as the terminal cell presides over a branch strain its immediate relatives remain quiescent. If it is destroyed, however, the sister at the subterminal position assumes the functional rôle of the lost cell.

A rather wide variation in the degree of regional correlation is suggested by those instances where a new terminal macrozoöid arises, not on the first branch as usual, but on the second or even third branch below the operated level.

The axial microzoöids on every branch do not differentiate into ciliospores. Loci of metamorphosing zoöids occur on about every third or fourth branch. The prospective value of a single axial microzoöid ($1X$) is predictable at a relatively early period by a marked growth in size. The growth may be taken as a criterion of at least a partial differentiation, to be completed a good many hours later

by a further increment in volume, modification of form, appearance of motile organelles, etc. A peculiar characteristic of these large cells is that they may differentiate directly into mature ciliospores or they may divide, giving origin to two zoöids of unequal size, both larger than any of the common types. The larger of the two matures first; the smaller grows to the size of its predecessor before metamorphosing or dividing again to produce two mature ciliospores in succession.

Regenerates sometimes arise from either those axial microzoöids which are, to all appearances, not predestined to metamorphose, or from those already partly differentiated. In the latter a potency or potencies in the process of expression apparently can be altered or superseded by others whose "urgency" toward expression is greater. The directional change in the process is referable to stimuli arising from the altered colonial organization. This is but another bit of evidence to the effect that cellular organization is dynamic and labile at certain periods and that changes going on within the cell which lead to recognizable morphogenetic characteristics are not necessarily irreversibly determined in direction. Many cases are known among the Protozoa where extrinsic or intrinsic factors lead to periods of reorganization, varying in considerable degree for the different groups and at different periods in the life cycle. In *Zoöthamnium alternans* we have a case where the re-direction of morphogenetic processes can be traced to an extrinsic cause: cutting the colony in the near vicinity of the cell subsequently affected.

Several significant problems arise in connection with the variable response of the axial microzoöids. Why do regenerates sometimes arise from axial microzoöids (1*X* or descendants) when as a rule the new growths are derived from the terminal branch cells? Attempts to induce regulation from 1*X* cells by trimming away all other cells on the branch gave no positive or predictable results. Until the question is investigated further in *Zoöthamnium* we can only interpret the variable behavior in terms of other work. Some of the merotomy experiments on other protozoa are suggestive (Calkins, 1911*a*, *b;* Peebles, 1912; Young, 1922; Dembowska, 1926; Taylor, 1928; and others). With respect to regenerative capacity these investigators were able to demonstrate progressive physiological changes in the cellular organization during the inter-mitotic period. Fragments cut at successive intervals after fission gave an increasingly high percentage of perfect regenerates. In *Uronychia* (Calkins, 1911*a*) even an emicronucleated fragment regenerated when cut immediately before the onset of fission. But in nearly all of the different forms studied the regenerative tendency disappeared sometime during the division process.

Another line of investigation summarized by Calkins (1934) and Summers (1935) demonstrates the cytological changes in cell organelles coincident with the division process. The resorption of old and the reappearance of new motor organelles, macronuclear reorganization, etc. suggest a brief period of cellular de-differentiation. There are probably analogous processes of alternating differentiation and de-differentiation in the history of the individual zoöids in *Zoöthamnium*. Time may be one of the important factors in individual cell behavior in relation to the balance between extrinsic coördinating influences and the aggregate of intracellular activities. That is to say, the intracellular activities of a *Zoöthamnium* cell may lead to the "fixation" of specific potencies at some critical period after cell division or, conversely, a cell may be more susceptible to the coördinating influences during or immediately after a division process. The axial microzoöid, for example, divides in from 20 to 70 hours after its derivation from the initial branch cell, whereas the terminal cell on the branch generally divides at intervals of about 12 hours. If a decapitation is made at a moment when the axial microzoöid is in some phase of divisional reorganization and the terminal cell in a more stable condition, the former instead of the latter may be activated or excited to prolonged generative activity. The supposition should be tested by a series of accurately timed operations above some particular branch.

An explanation of morphogenetic processes in *Zoöthamnium alternans* in terms of embryonic segregation at the time of division has already been attempted by Fauré-Fremiet (1930). In order to outline several points for discussion it is essential to review briefly his cytological analysis of normal development in this species. First, as regards the early axial divisions, the first three generations along the primary axis are unequal divisions. The inequality of the resulting daughters is reflected in the assortment of macronuclear material; each time the zoöids which remain in the terminal position (*TM*. 1, 2, 3) receive a larger portion of the macronuclei than the smaller branch microzoöids (*A*, *B*, *C*). On the supposition that the enlarged end of the macronucleus apportioned to the terminal macrozoöids represents a kind of segregation of chromatin material, these three unequal divisions are described by Fauré-Fremiet as qualitatively and quantitatively differential divisions. Beginning with the fourth division (division of *TM*. 3) the extremities of the dividing macronuclei in all later axial divisions are similar in size but in each instance a bit more of the finely striated mid-portion of the macronucleus is received by the zoöid remaining in the terminal position. All of these later divisions are characterized as quantitatively differential only. With

respect to the branch generations, all divisions along branches *A*, *B*, and *C* are similar and almost equal. The initial zoöids of subsequently produced branches (*D*, *E*, *F*, etc.) undergo qualitatively differential divisions: the axial microzoöids (1*D*, 1*E*, 1*F*, etc.) receive a greater share of the macronuclei than their lateral sisters (1*d*, 1*e*, 1*f*, etc.) although the cytoplasm in each case is distributed equally. The axial microzoöids (1*D*, 1*E*, 1*F*, etc.) represent the ciliospore-producing members of the colony. They undergo marked growth in size accompanied by a disintegration and reconstitution of the macronuclei which, although not described in detail, is characterized as an endomictic process. Fission in the lateral sisters (1*d*, 1*e*, 1*f*, etc.) is of no further interest cytologically; these zoöids constitute the main branch strains.

From the foregoing description it follows that differential division occurs at two points in the formation of all branches above *C*. To illustrate (Fig. 9): *D* receives less cytoplasm and macronucleus than

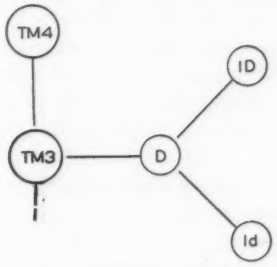

FIG. 9.

TM. 4, but the macronucleus in both resulting zoöids is qualitatively similar. The division is therefore quantitatively differential. When the initial branch cell *D* divides its cytoplasm is distributed equally but the macronucleus is assorted differentially because a thickened granular part goes wholly to the axial microzoöid 1*D*. The division is therefore qualitatively differential.

According to Fauré-Fremiet, "It appears clearly then that during the growth of a colony of *Zoöthamnium alternans* the two cells resulting from a division of one initial cell are never equivalent as to their 'potentialities.'" Also, "The character of the differential divisions on the main strain seems to determine the individual's differentiation of the colony; this differentiation depends not only on the individual's size, but also upon its physiological potencies." The differential divisions also appear to determine the characteristic features of the

median individuals and of the microzoöids. The latter have a limited power of growth and multiplication. Median microzoöids on branches *A*, *B*, and *C* remain as common nutritive zoöids, whereas the corresponding zoöids or their descendants on branches above *C* may undergo considerable growth and metamorphose into ciliospores.

Is it to be inferred from this analysis that quantitatively differential divisions determine (restrict) the subsequent power of division in branch strains and, further, that qualitatively differential divisions effect a segregation of potencies for ciliospore formation?

Regarding the first inference, all alternating median microzoöids, initial branch cells, are the lesser products of quantitatively differential divisions; they can divide only so many times, according to the number of generations normally produced on the branches of which they represent the beginning. While there is no doubt about the inequality of the cytoplasmic distribution between terminal macrozoöids and initial branch cells for the first ten generations or more, the inequality diminishes beyond this point until the two daughters are no longer differential as regards volume of cytoplasm. An equality may be achieved as early as the tenth and not later than the twentieth axial generation. Then what of those colonies that developed eight to fifteen generations beyond the twentieth node with similar axial-lateral relations? Another point to be made pertains to the regulative capacity of the branch zoöids. Those distributed in alternate positions along a branch axis seldom divide further so long as the integrity of the whole colony is preserved. When an apical piece is cut away from the colony some one of the more lateral zoöids on the remaining portion is capable of assuming the principal generative functions for relatively long periods of time. This behavior is difficult to interpret on the assumption that mitotic or "growth" potentialities are conditioned at either or both of the first two generations on the regulating branch.

The second inference may be challenged upon the grounds that segregation of ciliospore potencies does not occur at the specified division. Fauré-Fremiet adduces cytological evidence of segregation at the first three axial divisions and thereafter at the division of the initial branch zoöids. The latter is the fission at which the ciliospore-forming zoöids are separated from the main branch strain. In the light of newer findings, the restriction of ciliospore formation to 1*D*, 1*E*, 1*F*, etc. can be questioned. Ciliospores were observed to develop from axial microzoöids on the second and third branches, and also from both daughters of the fifth and tenth generations on branches *D* and *H* respectively of control colonies. Moreover, ciliospores oc-

curred on the regenerate in nearly every case of regulation from branch zoöids lateral to the supposed differential division provided, of course, that they were maintained for a sufficient length of time. The final bit of evidence comes from the demonstrated regulative capacity of the axial microzoöids (1*X*) on some of the operated colonies. When activated these were able to regenerate comparatively large sections of colony upon which all classes of zoöids except gamonts appeared.

The spatial relationship of cells should not be minimized as an important determining factor in an organism whose cells are so characteristically placed. The importance of this factor in development cannot be properly valuated until the general physico-chemical nature of the integrating mechanism and the medium through which it operates, presumably the neuro-muscular cord, are more fully understood. It is fairly certain, however, that it is not a specific factor, for in normal uncut colonies ciliospores occasionally develop in odd positions, and the microgamonts are apt to differentiate from the common zoöids at almost any position lateral to the axial microzoöids. Of related interest is the work of Buchanan (1927) on the flatworm *Phagocata*. The region from which a piece is taken with reference to the mouth of the intact worm is of no significance in determining the location of the mouth in the regenerate. Seyd (1935), on the other hand, reported a definite degree of regional specificity in the regeneration of a new mouth in *Spirostomum;* mouths in abnormal positions in the cut organisms degenerated and new ones formed at the correct locations.

The conjugation processes in *Zoöthamnium arbuscula* (Furssenko, 1929) and *Z. alternans* do not differ in essential detail. In the former the zoöids at the terminal position on each of the two primary axes (A_c and B_c) becomes the "macroconjugants." Until metagamic divisions occur, further growth along these axes is arrested. Two metagamic divisions of the conjugant result in a cluster of four large zoöids, two of which (A_2 and C_2) metamorphose directly into macrozoöids (ciliospores). The remaining two (B_2 and D_1) divide again, each giving rise to another macrozoöid and a stem cell. A stem cell produces an additional macrozoöid and a new (ex-conjugant) axis. A single conjugant therefore produces two ex-conjugant axes and from four to six successively produced macrozoöids.

The behavior of small lateral branches from the main axis at nodes basal to the conjugant in *Z. arbuscula* compares favorably with the precocious development of subordinate branches on a conjugating colony of *Z. alternans*. One or two of the small lateral branches (Seitenätschen) below the conjugant develop to the proportions of

regular main branches. In this fashion normal colonies with seven main axes are transformed, after conjugation, into colonies with nine to eleven chief axes. Furssenko refers to the new growths as "compensation" branches (Ersatzzweigen). It is his belief that the enormous growth of the macrozoöids occurs at the expense of food obtained from nearby microzoöids and, similarly, the compensation branches are destined to supply the energy needs of the ex-conjugant derivatives, i.e. the five or six clustered macrozoöids and the new ex-conjugant axes.

The chief support of Furssenko's hypothesis that ex-conjugant generations develop at the expense of adjacent regions comes from two observations: (*a*) the ex-conjugants themselves are not active feeders, and (*b*) the macrozoöids in neighboring regions either fail to mature or they divide to form common zoöids. The idea presupposes a mobilization and free transport of nourishment to regions active in development.

The observations on *Zoöthamnium alternans* are not in any way contrary to a possible rôle of the stalk in transportative phenomena. It is also quite likely, although yet to be proved, that nutrient materials are utilized by some zoöids at the expense of adjacent ones; this may be a cause contributing to growth inhibition or differentiation in nearby cells. Nevertheless it does appear that the precocious development of subordinate branches in a conjugating colony of *Z. alternans* is not primarily directed toward nutritional ends. In the first place, the actively developing apices of branch strains have energy requirements which, when taken as a whole, undoubtedly exceed the demands of the single ex-conjugant or its first few non-feeding descendants. The flux would therefore be directed away from the conjugant node. Secondly, in nearly every instance recorded the unusual development of the subordinate branches was well under way before the first post-conjugant division occurred. It is problematical whether or not the change in food requirements coincident with the transformation of a terminal macrozoöid into an ex-conjugant is sufficiently great to account for the relatively far-reaching alteration of the growth pattern.

Summary

1. *Zoöthamnium alternans* is a colonial protozoan of a rather special type whose constituent cells collectively possess in some degree many of the attributes of an integrated organism. Some of the integrating factors can be described in general terms from the work undertaken on form regulation.

2. When the apical cell of the primary axis is dissected away from a developing colony, a cell on some inferior branch, usually the first

below the cut, will differentiate into a new apical cell. The geographical limits within which positive regulative responses occur are given in the text.

3. Development of a colony continues from the newly differentiated apical cell. The structural and developmental characteristics of the normal colony persevere in the regenerated portion.

4. Evidence is presented to the effect that zoöids retain, for a time at least, greater developmental potentialities than are actually expressed when they comprise a part of the intact colony.

5. Under varying physiological conditions in the apical region of a colony, the coördinating influences exerted upon the mitotic activity of neighboring zoöids may be inhibitory (as shown by the responses evoked after decapitation) or excitatory (when the terminal macrozoöid is transformed into an ex-conjugant).

6. In the light of observations presented, the idea of dichotomous segregation or sifting out of potencies at fission is inadequate as an explanation of localization in this species. The experimental data do not confirm Fauré-Fremiet's cytological account of qualitatively differential divisions at specified division nodes on the branches.

7. There is cause to suspect that morphogenetic processes in particular zoöids of *Zoöthamnium alternans* (e.g. the presumptive ciliospores), once initiated and partly expressed in visible structure, can be conditioned or modified by cuts made in some neighboring region.

8. An hypothesis is offered to account for the origin of a regenerate from one or the other of several dissimilar cells of a branch strain. The explanation is based upon the factor of time in relation to the balance between extrinsic influences and the aggregate of intracellular metabolic activities by which potentialities are realized. The cells are thought to be more susceptible to external control during the reorganizational period of mitosis. There may be a critical time in cellular differentiation beyond which the intrinsic processes are not influenced by stimuli arising in some other part of the colony.

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